Transcriptional Profiling Reveals Barcode-Like Toxicogenomic Responses In The Zebrafish Embryo

L. Yang
Institut für Toxikologie und Genetik

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Gutacher: Prof. Dr. Uwe Strähle
Prof. Dr. Thomas Braunbeck

Abstract

There is an increasing demand by regulators, pharmaceutical manufacturers and industry for reliable and ethically acceptable model systems to assess and predict the toxicity of pharmaceutical products, chemicals, and waste. In particular, developmental toxicity of compounds has largely been ignored in the past raising massive concerns. While cell lines have great merits as test systems, they do not reflect the complexity of a developing embryo. Zebrafish and their embryos have been used in toxicity assays in the past and have a great potential for studying reproductive toxicity and the molecular basis of developmental toxicity.

Toxicogenomics is a powerful tool for compound classification based on mechanistic studies, which could eventually lead to the prediction of the toxicity of novel compounds. I have carried out here a systematic toxicogenomic study of zebrafish embryos with the aim to develop this system further as a model for molecular developmental toxicity studies. Questions such as stage- and compound specificity were addressed. The toxicogenomic responses to a series of 6 test compounds were highly stage-dependent. Moreover, exposure of late embryonic stages between 96 and 120 hours post-fertilisation induced transcriptional profiles that are characteristic for specific compounds. This leads to the identification of 199 genes that are induced by at least one of the 11 compounds including a number of signature genes that appear specific for individual compounds or compound groups within the set of chemicals investigated. Moreover, I have tested whether one can observe toxicogenomic responses in the absence of apparent morphological effects. In several instances robust gene responses were measured at concentrations that did not have obvious morphological effects. This raises hopes that toxicogenomic studies in the zebrafish embryo could be used to predict chronic effects of low level exposure. In situ hybridisation of a number of selected genes showed that the responses can be highly tissue restricted indicating that the whole mount protocol developed here is highly sensitive.

In conclusion, this toxicogenomic analysis, demonstrated that zebrafish embryos may be suitable to study the transcriptional responses to toxicants with high specificity and more sensitivity. The zebrafish may thus be developed into a system that will not only help to elucidate the molecular mechanisms of toxicity but may be also useful to predict the developmental toxicity of novel chemicals by comparison of toxicogenomic profiles.

Zusammenfassung

Der Bedarf seitens Regulatoren, pharmazeutischer Hersteller und der Industrie an verlässlichen und ethisch vertretbaren Modellsystemen zur Bewertung und Vorhersage der Toxizität pharmazeutischer Produkte, Chemikalien und von Abfall steigt beständig. Insbesondere die Entwicklungstoxizität von Verbindungen wurde in der Vergangenheit weitestgehend ignoriert, wodurch massive Bedenken aufkamen. Zelllinien besitzen eine hohen Stellenwert als Test Systeme, reflektieren aber nicht die Komplexität eines Embryos. Zebrafische und deren Embryonen wurden bereits für Toxizitäts-Assays eingesetzt und besitzen großes Potential, um Reproduktionstoxizität und die molekulare Grundlage der Entwicklungstoxizität zu untersuchen.

Die Toxikogenomik liefert ein leistungsfähiges Instrument zur Verbindungs-Klassifizierung basierend auf mechanistischen Studien, wodurch letztlich eine Toxizitäts-Prognose neuer Verbindungen möglich wird. Ich habe in der vorliegend Arbeit eine systematische toxikogenomische Studie an Zebrafischembryonen mit dem Ziel durchgeführt, dieses Testsystem zu einem Modell für Studien molekularer Entwicklungstoxizität weiterzuentwickeln. Fragen der Spezifität bezüglich Stadium und Verbindung wurden behandelt. Die toxikogenomische Antwort auf eine Serie von 6 Verbindungen erwies sich als hochgradig abhängig vom Stadium. Darüber hinaus wurden nach Exposition später embryonaler Stadien zwischen 96 und 120 Stunden nach der Befruchtung Transkriptions-Profile induziert, welche charakteristisch für spezifische Verbindungen waren. Dies führte zur Identifizierung von 199 Genen, die zumindest durch eine von 11 Verbindungen induziert wurden, inklusive einer Reihe von Genen, welche sich als spezifisch für individuelle Verbindungen oder Verbindungs-Klassen erwiesen. Weiterhin habe ich untersucht, ob die Beobachtung einer toxikogenomischen Antwort ohne sichtbaren morphologischen Effekt möglich ist. In einigen Beispielen konnten stabile Gen-Antworten in Konzentrationsbereichen gemessen werden, bei denen keine offensichtlichen morphologischen Effekte auftraten. Dies lässt hoffen, dass toxikogenomische Studien an Zebrafischen-Embryonen zur Prognose chronischer Toxizität nach Exposition mit geringen Konzentrationen eingesetzt werden können. *In situ*-Hybridisierungen von einer Reihe ausgewählter Gene haben gezeigt, dass die

Antworten hohe Gewebespezifität zeigen, was ein hohes Mass an Sensitivität des hier entwickelten „whole-mount“ Protokolls indiziert.

Schließlich zeigen die toxikogenomischen Analysen, dass sich Zebrafisch-Embryonen zur Untersuchung transkriptioneller Antworten auf Toxine mit hoher Spezifität und Sensitivität eignen. Der Zebrafisch kann daher möglicherweise zu einem System entwickelt werden, dass nicht nur bei der Aufklärung der molekularen Toxizitäts-Mechanismen hilft, sondern auch in der Prognose der Entwicklungstoxizität neuer Chemikalien durch Vergleich von toxikogenomischen Profilen nützlich sein kann.

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Abbreviations

A	adenosine
Cy3-dUTP	5- amino-propargyl-2'-deoxyuridine 5'-triphosphate coupled to cyanine 3
Cy5-dUTP	5- amino-propargyl-2'-deoxyuridine 5'-triphosphate coupled to cyanine 5
DIG	digoxigenin-11-UTP
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DTT	dithiothreitol
EDTA	ethylenediamine-N,N-tetracetate
G	guanosine
mg	milligram
µg	microgram
ng	nanogram
Hepes	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
hpf	hours past fertilization
L	liter
ml	milliliter
µl	microliter
mM	millimolar
µM	micromolar
mRNA	messenger RNA
nt	nucleotide
RNA	ribonucleic acid
rpm	round per minute
SDS	sodium-lauryl-sulfate
SSC	saline-sodium citrate buffer
T	thymidine
TE buffer	Tris/EDTA buffer
Tris Cl	tris(hydroxymethyl)aminomethane

1. Introduction

We are confronted with a steadily increasing number of compounds including pharmaceuticals, industrial products or waste with numerous potential sources of exposures. The impact of anthropogenic compounds on ecosystems and human health is an urgent and international issue since there is an ever-increasing number of examples of environmental disturbance, likely to affect biota and human health. Synergies and longterm low-dose effects, between this plethora of distinct chemicals increase the complexity or the potential toxic effects on the environment and human health.

Classically, conventional toxicology methods assess potential adverse health outcomes resulting from chemical exposure by using gross endpoints such as morphological or body and organ weight changes and histopathological observations. However, the analysis of histopathological or biochemical markers provide less information about a toxicant's mechanism of action. In addition, gross pathological changes are insensitive for detecting toxicity at low doses or early time point leading to incorrect assessment of potential toxicity or safety. In order to better evaluate the hazard associated with exposure to chemicals, we need to understand the specific mechanism of action of a toxicant. We are, however, far from a comprehensive understanding and assessment of chemical toxicity.

The number of molecular studies aimed at understanding the mechanism of toxicity is relatively small. ``Much of the so-called mechanistic research to date has been descriptive, whether at the level of the whole animal, the cell or at the molecular level. But in fact, it is still largely describing the parts and phenomena`` (Moore MN., 2002). These are not really addressing the core problem of how complex molecules interact with and are affected by contaminant chemicals. Changes in regulatory laws have created the need to assess the toxic effect of thousands of chemicals. Effective methods to elucidate the molecular mechanism of toxicity and eventually make predictions of the toxic potential of novel compounds have to be developed.

1.1 Toxicogemomics

The term toxicogenomics has been described as 'an emerging discipline that combines expertise in toxicology, genetics, molecular biology, and environmental health to elucidate the response of living organisms to stressful environments (Ramos., 2003). The '-omics' part of toxicogenomics also encompasses several other types of profiling technologies including protein profiling and metabolite profiling in a cell or tissue. The applications for toxicogenomics are diverse and include either mechanistic or predictive goals. Microarray-based approaches have the well-touted potential to be used for predictive toxicology with the ever expanding availability of large data sets of toxicogenomic data (Hamadeh et al., 2002; Pennie et al., 2001).

A novel toxicant may be identified by comparing the expression pattern it elicits with the expression patterns of known chemically induced expression 'signatures'. There is no doubt that toxicogenomic applications will lead to more effective and safer drug development, and will play an ever-increasing role in regulatory decisions leading to improved human health. There are challenges in using toxicogenomic approaches for mechanistic-based studies. As one pores over reams of toxicogenomic data, it is extremely difficult to glean mechanistic explanations linking the chemical exposure to the observed alterations in proximal and distal gene expression changes. Data enrichment through novel bioinformatics techniques has great potential, however, there are currently insufficient data sets to explain ever the simple chemically induced transcriptional responses. It is considerable more difficult to rationally explain the complex biological response by the genes found altered by a given chemical or drug. But the identification of chemically mediated gene expression changes that are associated with observed toxicities at multiple life stages within and across species, will likely get us closer to important players in the toxic response. The identification of these genes will open up opportunities for molecular and genetic based studies.

The measurement of gene expression levels upon exposure to a chemical can both provide information about the mechanism of action and form a sort of genetic signature from the pattern of gene expression changes. Furthermore the development of such gene

expression signatures would allow fast screening of unknown or suspected toxicants on the basis of their similarity to known toxicants (Lettieri., 2006).

1.2 DNA-microarray

The field of DNA microarray has evolved from EM Southern's key insight (Southern 1975) showing that labeled nucleic acid molecules could be used to interrogate nucleic acid nucleotides attached to a solid support. The Southern blot is considered to be the first DNA array (Southern 2000). Essentially the availability of the complete human and several other organisms (Drosophila, mice, and fish) genome sequences made the high throughput DNA array to become a practical method. Additionally, several new and highly sensitive techniques have been developed to promote the DNA microarray application. The first was the use of nonporous solid support, such as glass, which has facilitated the miniaturization of the array and the development of fluorescence-hybridization detection (Lochhart et al., 1996; Schena et al., 1995,1996). The second critical innovation was the development of methods for high-density spatial synthesis of oligonucleotides, which allows the analysis of thousands of genes at the same time. Third some specific mathematic analysis methods and software have been developed, which are used for microarray data analysis.

Nucleic acid microarrays use short oligonucleotides (15-25 nt), long oligonucleotides (50-120 nt) and cDNA (100-3,000 nt) as array elements. The short oligonucleotides are used primarily for the detection of single-nucleotide polymorphisms. The cDNA elements are readily obtained form cDNA libraries and are typically used for organisms for which only a limited part of the whole genome information is available. The oligonucleotides or cDNA are spotted onto solid support, such as glass, by using high speed robotics. Sample detection for microarrays on glass involves the use of probes labeled with fluorescent nucleotides. Fluorescent probes are generated from control and test RNA samples in single round reverse transcription reactions or amplified samples from limited start materials in the presence of fluorescently tagged dUTP or dCTP (Cy3-dUTP and Cy5-dUTP), which produces control and test products labeled with different

fluorochromes. The cDNA generated from these two populations, collectively termed the probe, are then mixed and hybridization to the array under a glass coverslip (Nuwaysir et al., 1999). The fluorescent signal is detected by using a scanner and data are analyzed with some specific analysis software that determines for each spot feature the ratio of Cy3 and Cy5, corrected for the local background.

Although there is tremendous potential for proteomics, and metabolomic approaches, with the state of current technology, microarray based gene expression profiling is the most feasible means by which to rapidly, and cost effectively, interrogate the sum biological response to chemical and drug exposure. DNA microarrays provide a great platform to perform genome-wide gene expression analysis through comparison of virtually and two biological samples. The limitations of DNA microarray are its ability to study only the mRNA levels and are not as sensitive for detecting small changes in gene expression.

I exposed zebrafish embryos at different developmental stages to a range of different toxicants and measured the changes in gene expression profiles by hybridization of cDNA to an oligonucleotide microarray. The overall aim was to establish the zebrafish embryo as a toxicogenomic sensor for monitoring environmental toxicants. To provide some knowledge for my study, this chapter presents the basic information of DNA microarray and toxicogenmics; adverse effects of toxins; the advance of molecular mechanism of these toxins. Later I will finally define the aim of my work more precisely.

1.3 Model organism selection

If genetic approaches are to be utilized to understand the mechanism of toxicity, simple whole organism systems such as the nematode, fruit fly or zebrafish may offer powerful advantages. First, these models enable rapid assessment of integrative system-level effects of chemicals and drugs. The scientific validity of using these system-based models developmental and neurotoxicitase conserved across species. This coupled with the fact is based on the assumption that the mechanism of genomes of these three

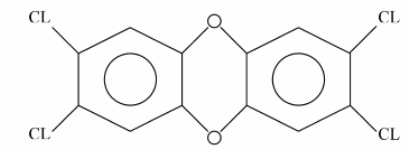
organisms have been completely sequenced, provides the potential to integrate developmental toxicity studies at the biochemical, cellular and molecular levels with observations at the structural and function level. This, in turn, will facilitate the construction of a genomics database for use in predicting developmental toxicity potential across species at the most fundamental level of homology. Of the three systems-based models, zebrafish exhibit several characteristics not shared by nematodes or flies that we believe make the zebrafish more immediately useful for developmental toxicity evaluations. For example, there are significant differences in genome structure between vertebrates and invertebrates (Postlethwait et al., 1998; 2000). Thus, while genes may share functional homology across species, mechanisms of gene regulation may differ significantly between invertebrates and vertebrates. Also, invertebrate physiology does not reflect the organization and size of that in vertebrates and so precludes direct comparisons between invertebrate and vertebrate toxicity. In contrast, the overall organization of the major components of the fish are highly homologous to humans (Rubinstein., 2003). The zebrafish also possesses all of the classical sense modalities, including vision, olfaction, taste, touch, balance and hearing, and their sensory pathways share an overall homology with humans. Another major advantage of the zebrafish is the embryos develop externally and are optically transparent. Thus, using simple microscopic techniques, it is possible to resolve development at cell resolution in vivo across a broad range of developmental stages. Resolution is increased by using transgenic zebrafish models that express fluorescent reporter genes, thereby making it possible to visualize dynamic changes in gene expression and detailed morphogenetic movements as they occur in the live, developing embryos (Blader et al., 2003). Several additional features of zebrafish biology, such as small size, rapid embryonic development and short life cycle (Dodd, et al., 2000; Spitsbergen et al., 2003; Zon et al., 2005), make this model system logistically attractive for developmental toxicity studies. Zebrafish reach sexual maturity within 3-4 months, reproduce year round, and produce clutch sizes that range between 100-200 embryos per mating pair. Because zebrafish adults grow to an average size of 3-4 cm and are easy to maintain at high densities, the infrastructure and maintenance costs required for housing the large numbers of animals required for comprehensive studies is relatively low. Such features are favorable for adapting this model system to high

throughput assays. High throughput chemical screens have indeed proven feasible in zebrafish to identify novel developmentally active drugs (Peterson et al., 2004a, b). Fish have served as useful sentinels to detect environmental hazards, and as efficient, cost-effective model systems for mechanistic toxicology and risk assessment for many decades (Harshbarger et al., 1990).

1.4 Model toxicants

I have chosen a number of model compounds known for their environmental relevance. The compounds include 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), valproic acid (VA, $CH_3(CH_2)_4CO_2H$), methyl-mercury chloride (MeHg), cadmium chloride (Cd, $CdCl_2$ $2H_2O$), 1,1-bis-(4-chlorophenyl)-2,2,2-trichloroethane (DDT, $(ClC_6H_4)_2CHCCl_3$), 4-chloroaniline (4CA, C_6H_6ClN), arsenic trioxide (As, As_2O_3), lead chloride (Pb, $PbCl_2$), acrylamide (AA, $CH{=}CHCONH_2$), aroclor 1254 (PCB), tBHQ (tert-butylhydroquinone $(CH_3)_3CC_6H_3$-1,4-$(OH)_2$).

1.4.1 TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)



TCDD
2,3,7,8-tetrachlorodibenzo-*p*-dioxin

A keyword query of PubMed for 'TCDD' yields over 5,580 manuscripts. Perhaps the most well studied environmental contaminant is TCDD. Decades of research have indicated that TCDD is toxic to all vertebrates studied (Peterson et al., 1993) and it induces a broad spectrum of biological response, including disruption of normal hormone signaling pathways, reproductive and developmental defects, immunotoxicity, liver damage, wasting syndrome, and cancer (Mandal., 2005). Despite enormous investments, the mechanism by which TCDD and related compounds perturb normal homeostasis is largely unknown. The general understanding is now that most, if not all, of the toxicity is mediated by activation of the aryl hydrocarbon receptor (AHR) and its dimerization partner, the aryl hydrocarbon receptor nuclear translocator (ARNT) (Mimura et al., 2003; Rowlands et al., 1997). The AHR is a member of the basic helix-

loop-helix Per-Arnt-Sim (PAS) family of transcription factors (Huang et al.,1993). This family of proteins includes AHR, aryl hydrocarbon receptor nuclear translocators (ARNT, ARNT2, and ARNT3), hypoxia inducible factor-1a (HIF1-a), endothelia-specific PAS protein-1 (EPAS-1/HIF2-a), single minded (SIM) and others (Carney et al., 2006). Most of the effects of TCDD require activation of the AHR, which results in transcriptional activation or the repression of a diverse array of genes (Nebert et al., 2000; Puga et al., 2000). Several groups have established zebrafish as an outstanding model to evaluate TCDD toxicity and have demonstrated conservation of the AHR pathway. The essential roles for AHR2 and ARNT1 have recently been confirmed using morpholinos and mutant zebrafish (Prasch et al., 2003, 2006; Antkiewicz et al., 2006). Three signaling functions have been described for the AHR pathway: adaptation signaling that leads to upregulation of xenobiotic metabolizing enzymes, toxic signaling that leads to adverse effects from high affinity ligands, and developmental signaling that is required for normal development of certain organs and tissues. A number of associated factors including chaperone and transcriptional co-activator have also been identified, but their role in toxicity remains unknown, and most data have been collected from tissue culture studies (Hankinson., 2005). A number of gene expression studies have been conducted revealing that CYP1A1 is highly induced in target tissue. Whether induced CYP1A1 activity is directly related to the manifestation of signs of toxicity remains somewhat controversial. The mechanism of adaptive, toxic, and developmental signaling by AHR all require ligand activation of the receptor, tanslocation of AHR to the nucleus, and heterodimerization with ARNT (Walisser et al., 2004). How the AHR mediates such diverse biological responses through the same mechanism is not fully understood.

1.4.2 MeHg (methyl-mercury)

Mercury is ubiquitous in the global environment. It occurs in several chemical forms, including elemental mercury and both inorganic and organic mercury (ethyl-, methyl-, alkyl-, or phenylmercury) compounds. Approximately 70% of the mercury in the environment comes from anthropogenic sources, primarily emission from coal-fired electric power generation facilities and waste dumps (Mason et al., 1994). Mercury is

used in a variety of industrial applications and manufacturing processes and in medical devices such as sphygmomanometers. It constitutes 50% of dental amalgams and ethylmercury was used as a vaccine preservative. Increases in power plant emissions and industrial uses during the past 100 years have been accompanied by a 3-fold increase in environmentally available mercury. There is no question that mercury is a global environmental contaminant and the public is increasingly more concerned with potential consequences of exposure (Trasande et al., 2005). Human exposure to toxic levels of mercury vapor in adults causes the classic triad of erethism, tremor, and gingivitis (Clarkson., 1997). Children exposed to high levels of vapor, may exhibit a syndrome known as acrodynia or pink disease (Baughman., 2006), which can be rapidly fatal under high doses exposure. Chronic exposure causes central and peripheral nervous system damage, manifesting as a characteristic fine tremor of the extremities and facial muscles, emotional lability, and irritability.

According to the Agency for Toxic Substance and Disease Registry of the U.S.A Department of Health and Human Services, mercury is listed as the third-most frequently found (lead and arsenic are first and second), and the most toxic substance in the United States (Lyn Patrick., 2002). Eighty percent of inhaled elementary mercury vapor is absorbed and can cross the blood-brain barrier or reach the placenta (Ozuah., 2000). Elemental mercury and its metabolites have the toxic effect of denaturing biological proteins, inhibiting enzymes and interrupting membrane transport and uptake and release of neurotansmitters (Clarkson., 2002). Inorganic mercury is complexed with glutathione in the liver and secreted in the bile as a cysteine mercury or glutathione mercury complex. Chronic exposure to inorganic mercury salts primarily affects the renal cortex and may manifest as renal failure or gastrointestinal problems. There is an enormous public concern over the potential link between mercury exposure and the development of autism. Despite or perhaps because of the lack of solid data, public concern remains high.

1.4.3 As (Arsenic trioxide)

Arsenic is widely distributed in nature, being found in food, the soil, water and airborne particles. It derives from both natural and human activities (Tchounwou et al., 1999). More than 80% of arsenic compounds are used to manufacture products with agricultural applications such as insecticides, herbicides, fungicides, algicides, sheep dips, wood preservatives, dye stuffs and medicines. Furthermore, arsenic has been used as an anticancer agent in the treatment of acute promyelocytic leukemia (Rousselot et al., 1999). In addition, arsenic can accumulate in groundwater. It is estimated that several million people worldwide suffer the effects of chronic arsenic exposure resulting from the environmental release of arsenic (Centeno et al., 2002). Groundwater contamination provides the majority of worldwide arsenic exposure. Many epidemiological studies have confirmed that exposure to arsenic and its compounds can have adverse effects on human health. Inhaling arsenic can cause lung carcinomas in particular, while ingestion in the form of drugs or in food and water, can provoke skin, respiratory system, liver and bladder tumors, as well as diabetes, cardiovascular and neurological disease (Goering et al., 1999). The adverse effects of continuous chronic exposure led WHO to lower their recommendation to 10 µg/l. Arsenic taken up by the body is mainly in the inorganic form, either as arsenite [iAs(III)] or arsenate [iAs(V)] (Rossman., 2003). Arsenite [iAs(III)] is more toxic than arsenate [iAs(V)], probably due to its faster rate of cellular uptake (Vega et al., 2001). After entering the cell, arsenate [iAs(V)] is rapidly reduced to Arsenite [iAs(III)]. In humans, inorganic arsenic is metabolized by methylation followed by excretion in the urine of iAs as well as monothylated and dimethylated forms. Methylated species are excreted more rapidly than unmethylated species (Marafante et al., 1987). iAs (III) is methylated by enzymatic transfer of the methyl group from *S*-adenosylmethionine to iAs (III) to form monomethylarsonous acid [MMA(V)]. The enzyme utilizes thioredoxin and NADPH as a reductant. MMA (V) can also be reduced by glutathione-S-transferase omega class 1-1 (Zakharyan et al., 2001). Historically, the methylation of arsenic was considered to be a detoxification process (Vahter., 2002). However, recent studies show that the trivalent methylated forms MMA (III) and dimethylarsinous acid [DMA(III)] often exceed iAs (III) in cytotoxicity and genotoxocity. Trivalent forms MMA (III) and DMA (III) are more clastogenic than their pentavalent counterparts and are able to produce reactive oxygen species (Nesnow et al., 2002). To date, most

estimates of risk at low levels of exposure have been based on extrapolations from data on highly exposed population, however the risk at low doses of the arsenic-induecd carcinogenesis is controversial (Tapio et al., 2006).

1.4.4 Cd (Cadmium)

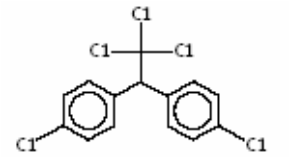
Cadmium is used in a variety of industries, e.g., nickel-cadmium batteries, electroplating, as a component of metallurgical and brazing-soldering alloys, in pigments, and as a stabilizer for plastic. Most of cadmium released in the environment occurs via a smelting other metals, notably zinc. Other sources of environmental cadmium are the burning of fossil fuels and waste materials, and the use of high phosphate and sewage sludge fertilizers (Ll'yasova et al., 2005). Environmental exposure to cadmium is a significant concern because it contaminates both soil and water at 534 superfund sites, and is ranked 7 on the EPA priority list of hazardous substances. The World Health Organization has shown that dietary cadmium exposure has a very wide range: inhabitants of worldwide nonpolluted areas have a daily dietary intake of approximately 40-100 μg, while inhabitants of polluted areas may obtain 200 μg or more as an average daily intake (Jarup et al., 1998). Between 10-50 percent of cadmium fumes are absorbed through the respiratory tract and approximately five percent of oral cadmium is absorbed through the digestive tact. Iron deficiency creates a significant risk for increased cadmium exposure by increasing gastrointestinal absorption from five percent to as much as 20 percent (Lyn Patrick., 2003). Cadmium exposure has been linked to cancers of the lung, liver, hematopoietic system, urinary bladder, stomach and pancreas in humans (Waalker., 2003). Epidemiological studies have found a positive correlation with elevated urinary cadmium levels and increased urinary calcium loss and elevated serum alkaline phosphatase levels, and also found a correlation between cadmium induced renal tubular damage and bone loss. In utero cadmium exposure studies in mice demonstrate that cadmium is embryotoxic, leading to mortality, cleft palate, limb malformations, exencephaly, and eyelid and tail malformations (Jeffrey et al., 1995). Studies in zebrafish reveal that cadmium is developmentally toxic, leads to localized cell death, alters muscle and motoneuron development and induces stress responses (Hen Chow et al., 2003;

Blechinger et al., 2002). The mechanisms of cadmium toxicity are not completely understood, but some of the cellular effects are known. Some of the specific changes that lead to tissue damage and death in chronic exposure have been related to oxidative stress and thiol depletion (Ercal et al., 2001). Cellular damage results from cadmium binding to sulfhydryl group in tissue, the production of lipid peroxides, and the depletion of glutathione.

1.4.5 VA (Valproic acid)

Valproic acid (VA) has been shown to possess multiple effects. It has been widely used for treating and preventing certain types of epileptic seizures for over twenty years (Ehlers et al., 1992), and is also used as mood stabilizer in bipolar affective disorders (Muller-Oerlinghausen et al., 2002), while VA is also known to have side effects during clinical treatment. The mechanisms underlying the therapeutic actions of VA are not well understood. One suggestion for the anti-epileptic function of VA is that VA prevents the stimulation of nerves by increasing the concentrations in the brain of the neurotransmitter, gamma-aminobutyric acid (GABA) (Johannessen et al., 2000). The common molecular mechanism is assumed to be depletion of inositol and inhibition of the collapse of sensory neuron growth cones in using VA in the therapy of bipolar disorders (Williams et al., 2002). During the clinical treatment, one major side effect of VA is teratogenictiy (Nau et al., 1991). With a probability around 1-2% women treated with VA during early pregnancy give birth to newborns with spina bifida aperta, a severe neural tube closure defect. In addition, numerous other subtle malformations are observed, such as defects of the heart, the skeleton and the facial skull. These major and minors defects are collectively called the fetal valproate syndrome (Huot et al., 1987; Ardinger et al., 1988; Martinez-Frias., 1991). The mechanism of VA teratogenicity remains unknown however, a recent toxicogenomic study using VA-exposed mouse embryos and embryocarcinoma cells has identified VA responsive genes (Kultima et al., 2004). Several of the identified genes are consistent with inhibition of histone deacetylase activity as being an important pathway modulated by VA.

1.4.6 DDT (bis[4-chlorophenyl]-1,1,1-trichloroethane)



DDT was first synthesized in 1874, and its insecticidal properties were described in the late 1930s. It was first used to protect military areas and personnel against malaria, typhus, and other vector-borne diseases. Commercial sales began in 1945, and DDT became widely used in agriculture to control insects, such as the pink boll worm on cotton, codling moth on deciduous fruit, Colorado potato beetle and European corn borer (Rogan et al., 2005). Its use was banned in Sweden in 1970 and in the USA in 1973, largely on the basis of ecological considerations, including persistence in the environment and sufficient bioaccumulation and toxic effects to interfere with reproduction in pelagic bird (Turusov et al., 2002).

In the environment, DDT breaks down to P,P'-DDE (bis[4-chlorophenyl]-1,1-dichloroethene), an extremely stable compound that resists further environmental breakdown or metabolism by organisms. DDE is the form usually found in human tissue in the highest concentration, especially in areas where there has been no recent use of the parent compound. The general population is exposed to DDT mainly through food, whereas occupational exposures are mainly through inhalation and dermal contact. DDT and DDE can also be transferred from the placenta and breastmilk to fetuses and infants. Acute exposure to a high dose of DDT can cause death in laboratory animals. The environmental impact of DDT is widely known and because of public outcry, importantly it continues to be widely used in other parts of the world and has been credited in saving millions of lives by reductions in malaria-related mortality (Schapira., 2004). Since the global burden of DDT persists, a mechanistic understanding of the mode of action of DDT remains an important goal. To date, a molecular explanation of the action of DDT on vertebrate development remains uncertain (Brandt et al., 1998; Eriksson., 1997).

1.4.7 4CA (4-chloroaniline)

Chloroanilines are widely used intermediate chemicals in the production of several herbicides, dyes, agricultural agents and many industrial compounds. Occupational, agricultural or contaminated waterways are potential routes of human exposure. Exposure to aniline or its derivatives results in several toxicities including hematotoxicity, splenotoxicity, hepatotoxicity and nephrotoxicity (Chhabra et al., 1990). Methemoglobinemia and hemolytic anemia appear to result from hepatic biotransformation of aniline or a chloroaniline via N-oxidation to phenylhydroxylamine metabolites which then mediate hematotoxicity (McMillan et al., 1990; 1991). Hematotoxicity may also be a contributing factor to the splenotoxicity induced by anilines. Toxicity studies in zebrafish demonstrate that 4-chloroaniline is embryotoxic, impacting liver, kidney and gills (Burkhardt-Holm et al., 1999; Oulmi et al., 1996). There is only limited toxicological data for this environmental contaminant. It is less clear how anilines induce the toxicity and what role the parent compound or metabolites might play in organ directed chloroaniline toxicity.

1.4.8 PCB (Polychlorinated biphenyls, Aroclor 1254)

Organochlorine contaminants remain a profound health hazard because these toxicants are resistant to biodegradation and remain widely distributed in the environment (Giesy et al., 1998). Polychlorinated biphenyls (PCBs) are mixtures of man-made chemicals with similar chemical structures. Due to their non-flammability, chemical stability, high boiling point and electrical insulating properties, PCBs were used in hundreds of industrial and commercial applications including electrical, heat transfer, and hydraulic equipment; as plasticizers in paints, plastics and rubber products; in pigments, dyes and carbonless copy paper and many other applications. More than 1.5 billion pounds of PCBs were manufactured in the United States prior to cessation of production in 1977. Polychlorinated biphenyls (PCBs) have been reported at alarming levels in the Arctic environment (De-Wit et al., 2004). Several studies have reported adverse effects associated with PCBs, including altered stress response (Jorgensen et al., 2002; Aluru et al., 2004), immune functions (Maule et al., 2005) and liver metabolic response (Vijayan et al., 2006). PCB exposure has been associated with cognitive and motor

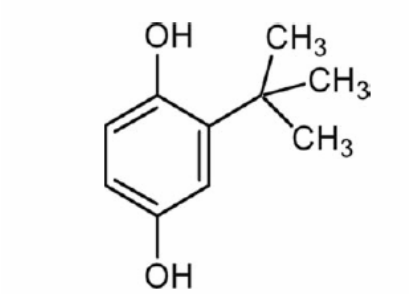
neurobehavioral abnormalities in both humans and experimental animal (Jacobson et al., 2003; Branchi et al., 2005). Dopaminergic system appears to be a cellular and neurochemical targets for PCBs (Bemis et al., 2004; Seegal, 2003; Lee et al., 2006). Aroclor 1254, which consists of more than 80 PCB isomers and congeners, is classified as a class A carcinogen. The ability of PCBs to act as a tumor promoter may be linked to deregulation of several pathways including overexpression of proto-oncogenes and other cell cycle regulators and may embarque on a non-genotoxic route of carcinogenesis. On the other hand, metabolic activation of PCBs causes oxidative stress and formation of DNA adducts. A further complication in an assessment of Aroclor 1254 toxicity is the established cross-talk of certain nuclear receptors with AHR-ARNT. Thus the conflicting information from animal studies and epidemiological finding may well be the result of a complex cross-talk amongst several orphan nuclear receptors and transcriptional regulators (Borlak et al., 2005).

1.4.9 Pb (Lead)

Lead is a heavy metal common in nature. Its abundance, joined to such physical characteristics as ductility, high density, low chemical reactivity, easy extraction, and low cost, have made it broadly used since prehistory. Environmental lead (Pb) contamination can be traced back 5000 years to Ancient Rome (Jarup et al., 2003). It has been used in medicines, paintings, pipes, ammunition, vitrified ceramic, and, in more recent times, in alloys for welding, chemical reagent storage, electric batteries, protection against ionizing radiation, and as an antiknock agent in gasoline (Garza et al., 2006). Although environmental lead pollution has now been reduced to a remarkable extent, lead poisoning still occurs (Sen et al., 2002). In the United States, lead paint is a primary source of lead exposure and the major source of lead toxicity in children. Drinking water is also a major source of lead exposure, estimated to be responsible for approximately 20 percent of the total daily exposure experienced by the majority of the U.S. population. One study associated the storing of food in lead-glazed containers with elevated blood lead levels (Rojas et al., 1994). The center for disease control currently considers lead poisoning the foremost environmental health threat to children in the United States (Lyn

Patrick., 2006). Data now indicates that low-level exposures resulting in blood lead levels below 100 µg/1 result in cognitive dysfunction, neurobehavioral disorders, neurological damage, hypertension, and renal impairment (Lyn Patrick., 2006). In addition, lead exposure may be a lifetime threat to certain lead exposed populations during pregnancy, lactation, and post-menopause (Gao et al., 2006). Furthermore, the carcinogenic potential of lead has recently been revised, leading to the conclusion that inorganic lead compounds are probably carcinogentic to human (Silbergeld., 2003). Lead is currently listed as one of the most hazardous substances by the Agency of Toxic Substances and Disease Registy. This aspect has roused once more the interest to the understanding of the specific mechanisms of lead actions.

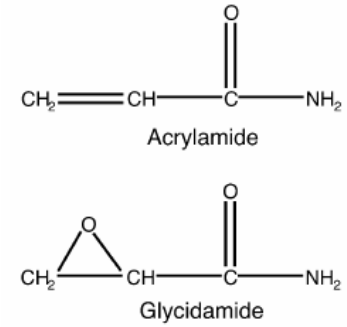
1.4.10 tBHQ (tert-butylhydroquinone)



Tert–butylhydroquinone (tBHQ) is used as an antioxidant in cosmetic products such as lipsticks, eye shadows, perfumes, blushers, and skin care preparations at concentrations ranging from 0.1% to 1.0%; the chemical is also used at concentrations up to 0.02% in oils, fats, and meat products to prevent rancidity, and as a polymerization inhibitor for various polyunsaturated polyesters (CIR, 1986). tBHQ is also a major metabolite of 3-tert-butyl-hydroxyanisole (BHA) in vivo in animals and humans (Nakamura et al., 2003). Both tBHQ and BHA have received a lot of attention due to their ability to induce phase II detoxification enzymes, including glutathione S-transferase and NAD(P)H-quinone oxidoreductase, and their potential role in cancer prevention (Li et al., 2002). tBHQ was nominated for toxicity and carcinogenicity testing by the Food and Drug Administration. The results (Natl Toxicol Program Tech Rep Ser. 1997) suggested that there was no evidence of carcinogenic activity of tBHQ in F344/N rats and B6C3F mice. Exposure of rats to tBHQ in feed resulted in decreased incidences of mammary gland neoplasms. Distribution and excretion studies showed that tBHQ was found in

kidney and liver tissue, the metabolites were found in urine when administered orally to Fischer 344 rats (Ikeda et al., 1998). A high dose (400 μmol/kg) glutathione conjugates of tBHQ, a metabolite of the urinary tract, however, were found to be toxic to kidney and bladder (Peters et al., 1996). tBHQ is cytotoxic for human monocytic leukemia cell through disruption of mitochordiral structure and decrease of intracellular glutathione and ATP (Okuboa T et al., 2003). In vitro studies (Gharavi et al., 2005) showed that tBHQ can directly modulate the expression of Cyp1a1 through an AHR dependent pathway by acting as an AHR ligand. Cell culture research manifested that exposure of rat and human hepatocytes to BHA and tBHQ can induce Heme Oxygenase 1 (HO-1) and NAD(P)H:quinine oxidoreductase I (NQO-1) correlated with activation ERK1/2 and JNK1/2 (Keum YS et al., 2006). The toxicity of tBHQ remains somewhat controversial according to in vivo and in vitro results.

1.4.11 AA (Acrylamide)



Acrylamide has received considerable attention recently due to its presence in virtually all baked, grilled or fried foods. Acrylamide, which is an important industrial monomer, has been manufactured in big scale since the 1950s (Rice., 2005) mainly to produce water soluble polyacrylamides used as flocculents for clarifying drinking water, for treating municipal and industrial waster waters and as flow control agents in oil-well operations. Other major uses of acrylamide are in soil stabilization, in grout for repairing sewers and in acrylamide gels used in biotechnology laboratories (Jägerstad et al., 2005). The general opinion has been that the main human exposure to acrylamide is of occupational origin in the industrial production of polyacrylamide, while the general public may be exposed by drinking water that has been treated with polyacrylamide in a refining process (Abramsson-Zetterberg A., 2003). A maximum tolerable level of 0.1 μg /l acrylamide in water has been established within the European Union (Jägerstad M et al., 2005). Data (Tareke et al., 2002) showed that the high background level of acrylamide in humans was due to compounds in the diet by demonstrating relatively high levels of

acrylamide in heat-processed foods principally by the Maillard reaction between the amino acid asparagines and reducing sugars (Mottram et al., 2002), especially in carbohydrate-rich foods such as crisps and French fries. Acrylamide is biotransformed *in vivo* to its epoxide, glycidamide, which is genotoxic in a variety of *in vitro* and *in vivo* test systems (Calleman et al., 1990). Acrylamide and glycidamide are detoxified by glutathione conjugation, and glycidamide is also detoxified by hydrolysis. Acrylamide can induce chromosomal aberrations, sister chromatid exchange and mitotic disturbances in mammalian cells in vitro. It induces structural chromosomal aberrations *in vivo* in both somatic and germ-line cells. Chromosomal aberrations and micronuclei were induced in mouse bone marrow and in premeiotic and postmeiotic cells in a linear dose–response relationship (Paulsson et al., 2003). The *in vitro* experiments showed that acrylamide is not mutagenic in bacterial cells; it is direct-acting mutagenic in mammalian cells. Based on the results of *in vivo* mammalian somatic cell assays, acrylamide appears to be clearly genotoxic, interfereing with spindle apparatus (polyploidy, aneuploidy) at relatively high doses (Carere., 2006). Several facts have confirmed and extended the earlier evidence that acrylamide is genotoxic *in vivo* and that genotoxicitiy is mediated by biotransformation of acrylamide to glycidamide. Cells treated with either glycidamide or acrylamide had more A→G transitions and G→C transversions than spontaneously mutated control cells, and glycidamide treated cells also had more G→T transversions (Besaratinia et al., 2004). Meanwhile there are clear increases in tumors in several organs after exposure of Fischer 344 rats to acrylamide (Friedman et al., 1995). However, findings of epidemiological studies on comparative cancer incidence in human populations having different intensities and durations of acrylamide exposure reveal no increased cancer risks (Mucci et al., 2003; 2004; Pelucchi et al., 2003). Additional research is ongoing on the carcinogenicity and genotoxicity of acrylamide in rats and mice. Only neurotoxicity has been seen in both animals and humans exposed to acrylamide (Shipp et al., 2006). Additional study on the model of action of the reproductive and carcinogenic effects in rodents is needed to determine the relevance of these observations to human health outcomes.

1.5 Molecular mechanism of toxicity

Previous studies in cells and animals have suggested that toxicants can interfere with all factors at many levels. The gene level of oxygen radicals with the concomitant oxidative stress responses is a very frequent effect. In addition, more specific interaction with signaling system and specific transcription factors, have been observed. Although there are lots of studies focused on the toxic effects, the mechanism through which the toxicants exert their toxicity, are in many cases still unclear.

1.5.1 Oxidative stress and DNA damage

In general, the most common modification of DNA occurs as a consequence of unquenched reactive oxygen species (ROS), most of which are generated during the reduction of molecular oxygen to water on the electron transport chain (ETC). When levels of ROS surpass the protective mechanism of a cell, they are capable of interacting with lipids, proteins, or nucleic acids (Moosmann et al., 2002). The occurrence of oxidative stress, caused by ROS, is believed to be a major etiological or pathogenic event of most degenerative processes. Several findings that neurotoxicants, e.g., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), dieldrin, paraquat, and rotenone are capable of eliciting neurodegeneration by disrupting various complexes in the ETC and increasing ROS production, supported the environmental toxin/oxidative stress theory of neurodegeneration (Betarbet et al., 2000; Corrigan et al., 2000).

1.5.2 Signal transduction

Signal transduction is the mechanism by which extracellular signals are transferred to the cytosol and nucleus of the cells. Growth factors, neurotransmitters, and hormones serve as first messengers and G-protein coupled receptors, growth receptors with tyrosine kinase activity, neurotransmitter receptor, and nitric oxide (NO) have function for second messenger systems. Any interference with these processes in early stages of development

would have the potential to have profound effects on the function of the cells as well as their development. In recent years, investigators have considered a number of signal transduction pathways as potential targets for toxins including metals, alcohols, and persistent environmental pollutants. Nine pesticides, 14 solvents, 10 metals or organometal compounds, and 6 other industrial chemicals were identified as developmental neurotoxicants (Anderson et al., 2000). Among metals, methylmercury and lead have been shown to affect several signaling pathways including calcium levels (Limke et al., 2003), calcium channels (Sirois et al., 2000), and protein kinase C (PKC) (Nihei et al., 2001). Ethanol has been shown to affect several signal transduction pathways such as cyclic AMP metabolism and phospholipids/PKC (Hoek et al., 1990; Hoffman et al., 1990). PCB (Polychlorinated biphenyls) can induce alterations in intracellular free Ca^{2+} (Kodavanti et al., 1993; 1994). The disruption of Ca^{2+}-homeostasis may have a significant effect on other signal transduction pathway.

1.5.3 Zinc finger transcription factors

Zinc finger proteins that are structural motifs found in several classes of proteins, were proposed to function in eukaryotic protein-nucleic acid interaction such as gene expression and DNA repair (Pabo et al., 1992; Beckmann et al., 1997). Several studies have shown that metal such as lead (Pb) and mercury (Hg) interfered with the DNA binding properties of a member of zinc finger proteins family, namely specificity protein one (SP1), and demonstrated that the effects of these metals were mediated through interaction with the zinc finger domain of SP1 (Zawai NH., 2004). Microarray analysis has shown that Oct-2, a member of POU family, was a potential molecular target of lead, and may thus mediate some of the effects of lead on neurotransmission (Zawai NH., 2004).

1.6 Aims of this project

A principal goal of this work is to establish genetic tools and techniques that will be used to monitor the environmental contaminants and will allow elucidation of the mechanism

by which environmental chemicals perturb normal vertebrate development. This work will pave the way for the systemic use of the zebrafish embryo in molecular toxicity and genetics.

First, I want to establish the zebrafish as a genetic model for monitoring the environmental contaminations. Using genome-wide microarray in zebrafish, I wanted to identify gene expression changes in response to a set of model toxicants.

Second, after the identification of these signature genes, I focus on a detailed expression of several selected genes that have been induced by toxicants.

2 Materials and Methods

2.1 Chemicals

Cadmium chloride ($CdCl_2 \cdot 2H_2O$), Methylmercury (II) chloride (CH_3HgCl), DDT, (1,1-Bis-(4-chlorphenyl)-2,2,2-trichlorethan) ($(ClC_6H_4)_2CHCCl_3$), 4-chloroaniline ($ClC_6H_4NH_2$), TCDD,(2,3,7,8-tetrachlorodibenzo-p-dioxin), Lead (II) chloride ($PbCl_2$), Valproic acid ($CH_3(CH_2)_4COOH$), Arsenic(III)dioxide (As_2O_3), Acrylamide ($CH_2=CHCONH_2$), Aroclor 1254, tBHQ (tert-Butylhydroquinone $(CH_3)_3CC_6H_3$-1,4-$(OH)_2$) were purchased from Sigma-Aldrich company. Other general chemicals supplied by Car Roth GmbH & Co (Karlsruhe, Germany).

2.2 Zebrafish (*Danio rerio*) embryos

Zebrafish wild type strains AB, ABO and Tübingen were kept and bred as described (Westerfield., 1993) in the fish facility of the Institute of Toxicology and Genetics, (Forschungszentrum Karlsruhe, Germany).

2.3 Enzymes

All restriction endonucleases and PCR enzymes were supplied by Promega (Mannhein, Germany) unless otherwise stated.

2.4 DNA oligonucleotides

All DNA oligonucleotides were synthesized by Metabion international AG (Martinsried, Germany).

2.4.1 DNA oligonucleotides for RT-PCR.

Cyp1a1 (AF057713): F. TGGATGAAAAGATCGGGAAG

R. GCCAGTTTTCCTGGATTTCA

Keratin (AI397347): F. GGCAGGTCGATCTGAATCAT

R. TTGACACCACTTGTCCATCG

BI533854: F. GCAGGAATTGGAACATGTCA

R. TTGCTTTGCTTTTATTGATTTGA

Parv3a (BE201681): F. CGGACACCACTCAGCCTTAT

R. TGTGCGCTCTCTACATGTCC

Mmp9 (AW174507): F. GCCCTGATCGTGGATACAGT

R. TTGCCTTTTCCTCTCTGCAT

ATF3 (AW422298): F. GGACGTGGAGATGACAAGGT

R. TCGGATGTCTTGTGGTGTGT

AY050500: F. ACGGTCAAACTTCTGCTGCT

R. AAGCGCTAGAGCTCCACAG

BI843145: F. TTCTGCATTTCTTCCGGGTA

R. ATGTTGCACTTTTGCTGACG

BI980610: F. CGCGAGTTCACTTTCATTCA

R. GTGTCTTTTCCAGGCTTCCA

Glutathione peroxidase I (AW232474): F. ATTTTTCGCGCCTCCTATTT

R . CCCGATGAGGAACTTTTCAA

BG727181: F. CGATTCCTCCTCCTTTTTCC

R. CTCTTGCTCCATCCTTCCAG

Mmp13 (AW305943): F. ACCAGGAGACACTGGAGGTG

R. AGGATAACTGCGCCAGAAGA

Hsp70 (AF210640): F. GGCACCACCTACTCCTGTGT

R. CACAAAGTGGTTCACCATGC

β-actin: F. CCCAGACATCAGGGAGTGAT

R. AGGAAGGAAGGCTGGAAGAG

2.4.2 Morpholino oligonucleotides

The morpholino oligomers were synthesized by Gene Tools (One Summerton Way, Philomath, OR USA).

mmp9: GAAACGCCAGGACTCCAAGTCTCAT
mmp13: AAGCAGGTCTTCATGTTTCTCGTGC

2.5 Kits

Total RNA extraction kit, DNA Gel extraction kit and NucleoSpin Plasmid kit (mini preparation) were supplied by Macherey-Nagel (Düren, Germany). The mRNA isolation kit was purchased from Ambion (Europe, Huntingdon, UK). The CyScribe first strand cDNA labeling kit was from Amersham Biosciences (Freiburg, Germany), and the plasmid purification kit (Max preparation) from Qiagen (Hilden, Germany).

2.6 Zebrafish (*Danio rerio*) Oligonucleotide library

Zebrafish oligonucleotides library contain 16,228 non-redundant probes was purchased from the Sigma-Genosys/Compugen and printed two replicated sub-arrays per chip in the Microarray facility (ITG, FZK).

2.7 General methods

2.7.1 Preparation of competent *E.coli* using $CaCl_2$ and transformation.

(Molecular Cloning 1,116-118)

2.7.2 In situ hybridization (Oxtoby et al., 1993)

2.7.3 RT-PCR (Sambrock et al., Edn 3rd edition 2001)

2.7.4 Microinjection (Stuart et al., 1990)

2.8 Exposure of embryos to chemicals

2.8.1 Solution Preparation of Chemicals

According to the information of these chemicals, the chemicals were dissolved in different solvents, such as, water, dimethylsulfoxide (DMSO), ethanol or toluene as stock solutions. Cd, 100 mg/l (H_2O); Hg, 100 mg/l (H_2O); DDT, 100 mg/l (0.2% ethanol); 4CA, 100 mg/l (0.2% ethanol); TCDD, 10 µg/l (28 mg/l toluene and 0.5% DMSO); VA, 100 mg/l (H_2O); As, 50 mM (H_2O); Pb, 62.5 mM (H_2O); AA, 0.5 M (H_2O); PCB, 50 mg/ml (ethanol); tBHQ, 200mM (ethanol).

2.8.2 Decision on the concentration of chemicals

Embryos were grown in embryo medium (60 µg/ml Instant Ocean, Red Sea Fish Pharm Ltd.). Using different concentrations treated embryos or larvae, according to the half lethality dose, reduce the concentration. Criteria of concentration decision: more than 50% of embryos or larvae show morphological changes after treatment for at least one of several morphological criteria at 48 hpf. Exposure of larvae to Cd, Hg, DDT, TCDD, 4CA and VA of 96 to 120 hpf at the same condition did not result in a strong morphological effect.

2.8.3 Method of exposure embryos or larvae to chemicals

Embryos of AB and ABO background were harvested as described by Westerfield, and exposure the embryos or larvae to different concentrations of chemicals at different stages, meanwhile keeping the control groups in fish water or vehicle solvents. I used petri dishs (135 mm) to culture embryos in 100 ml vehicle control at 28.5°C. Different numbers of embryos were exposed to the chemicals: between 4 to 24 hpf (600 embryos), 24 to 48 hpf (400 embryos) and 96 to 120 hpf (200 embryos). Embryo medium alone (Cd, Hg, Pb, As, VA, AA) or 0.2% ethanol in embryo medium (tBHQ , 4CA, PCB, DDT) or the definited volume of 0.5% DMSO, 28 mg/l toluene in embryo medium (TCDD) were used as vehicle controls.

2.9 Extraction of total RNA and mRNA

2.9.1 Total RNA extraction

After exposure, the embryos were collected and immersed immediately in liquid nitrogen. Frozen embryos were ground, then lysis buffer was added and vortexed vigorously. After filtration, RNA was bound to silica membrane and desalted. After digestion of DNA and washing several times, total RNA was eluted in nuclease-free water. Then 2.5 volumes of ethanol (100%), 0.1 volumes of sodium acetate (3M) were added to the RNA solution and left at -20°C overnight, or quick by frozen in -70°C for 30 minutes. Then I recovered the RNA by centrifugation and washed several times with 70% ethanol. Finally it was dissolved in 250 µl nuclease-free water. The integrity of total RNA was examined by denaturing agarose gel (FA) electrophoresis and the quantity was checked by DanoDrop spectrometer (DanoDrop technologies, Wilmingto USA).

2.9.2 mRNA extraction

To the total RNA, I added 250 µl 2×binding solution, mixed thoroughly and combined with oligo(dT) cellulose, incubated the mixture at 65-75°C for 5 minutes and 60 minutes at room temperature with gentle agitation. And then the mixture was washed several times with washing buffer and eluted by preheated RNA storage solution. Finally after precipitation and recovering, the pellet was dissolved in nuclease-free water at a 250-500 ng/µl final concentation.

2.10 Microarray

2.10.1 Preparing fluorescently labeled probe from mRNA

mRNA (1.5-2 µg), 1 µl random nonamers, 1 µl anchored oligo (dT), 1 µl spike 7 or 8 (1 ng/µl) and water to a total reaction volume of 11 µl in a 1.5 ml amber microcentrifuge tube. I mixed and incubated the reaction mixture at 70°C for 5 minutes. Then I let the mixture cool at room temperature for 10 minutes and spun down it shortly. Added 4 µl

5× CyScript buffer, 2 µl DTT (0.1 M), 1 µl Cy3 or Cy5 (25 nmol) respectively, and 1 µl reverse transcriptase (100 U/µl). The final reaction volume was 20 µl after addition of all the components. After mixing, the reaction was incubated at 42°C for 1.5 hours. Then 5 µl 1 M sodium hydroxide was added and incubated at 37°C for 15 minutes to hydrolyze the RNA. The cDNA was neutralized with 10 µl 2 M HEPES and mixed well.

2.10.2 Purification of probe with Microcon columns (Millipore Microcon YM-30)

I added 465 µl TE (pH 7.5) buffer to the reaction mixture and centrifuged at 9000 rpm for 12 minutes, turned the column, inserted it into a new microcentrifuge tube and centrifuged 4 minutes at 9000 rpm. The probes labelled with Cy3 and Cy5 were combined and 400 µl TE buffer were added, centrifuged at 9000 rpm for 12 minutes. TE buffer was added to elute probes to a final volume of 16.5 µl. 1.5 µl probe was used to determine the quality and quantity with a NanoDrop spectrometer.

2.10.3 Hybridization on Microarray

The probes were heated at 95°C for 2 minutes, cooled down on ice for 2 minutes and kept at room temperature for 5 minutes, then spun down and 15 µl DIG-easy hybridization buffer preheated at 42°C was added. The probes were put on the chips and cover slips were careful placed on top to avoid bubbles. The chips were placed in hybridization chambers and incubated at 42°C for 16-18 hours.

2.10.4 Washing and scanning chips

The chips that were hybridized overnight were put in wash buffer 1 (2×SSC, 0.1% SDS) and incubated at 42°C for 5 minutes; washed in buffer 2 (0.1×SSC, 0.1% SDS) at room temperature for 10 minutes with gentle agitation; washed in buffer 3 (0.1×SSC) 4 times

for 1 minute respectively and dipped into buffer 4 (0.01×SSC) shortly and dried by centrifugation.

Arrays were scanned using the Axon model 4000B dual-laser scanner and the corresponding GenePix 4 software (Axon, Union City, CA). Both channels (532 nm for Cy3 and 635 nm Cy5) were scanned in parallel and stored as 16-bit TIFF files. The absolute intensity values span the range from 0 to 64,535. The scans were performed with a resolution of 10 μm. From each spot with a mean diameter of 100 μm, 100 data pixels were recorded. Individual local background area around the spots were defined, which included ~400 pixels and excludes neighboring spots. For each channel, the raw data was calculated as the median intensity of all foreground pixels with respect to all background pixels. Each array was scanned three times (low, medium and high scan) with different signal amplification factors (voltage settings of the photomultiplier tubes), but with the same laser power. The channels for Cy3 and Cy5 were balanced in each scan for approximately the same intensity profile. In the low scan no spot was saturated; in the high scan the signal amplification for Cy5 was set to ~80% of maximum and Cy3 amplification was adjusted to this. The settings used in the medium scan lie between the low and the high scan. This method of scanning has several advantages. In the low scan where no spot is in saturation, it is possible to calculate the real ratio for genes levels with very high expression. In order not to loose these, a high scan is made; in this case, the information on saturated spots is lost, so the two scans complement each other. The medium scan produces additional values for subsequent calculation. By scanning the arrays three times, errors which occur while recording and which might increase the error factor in the normalization are averaged. However, signals from gene expressed at very low level will not be detected.

3 Microarray data analysis

3.1 Printing chips

The zebrafish oligonucleotides library was bought from Sigma-Genosys (The Woodlands. TX, USA) and Compugen (Jamesburg. NY, USA), with 43 plates (384 wells) including 16,228 modified oligonucleotides. Upon evaluation it turned out, however, that plates 29 to 43 had faulty anime linkers impairing the retention of the olignucleotides on the coat-link slides. As the companies were unable to replace the defective oligonucleotides, we used the reduced set of intact oligonucleotides (384-well plates 1 to 28). These oligonucleotides were printed into CodeLink activated slides (Amersham Biosciences, Europe) in the microarray facility at the Institute of Toxicology and Genetics using a multi-axis Omnigrid 100 spotter (San Carlos. CA). The zebrafish chip was arranged in 48 blocks with 12 rows and 4 columns (Figure 3.2). Each block was printed by one pin. Pins were organized in 2×12 pins (TeleChem SMP3 pins, Sunnyvale CA) in printhead to produce 27×27 spots in each block (Figure 3.1). The diameter of the spot is 100 µm and each oligonucleotide was printed twice. Before starting to print chips, the pins needed to be cleaned and subjected to a test print. After calibration and checking the quality of the test spots, 100 CodeLink slides were fixed on the printboard and the pin parameters were adjusted and set up in the computer, finally click the mouse and run printing programme.

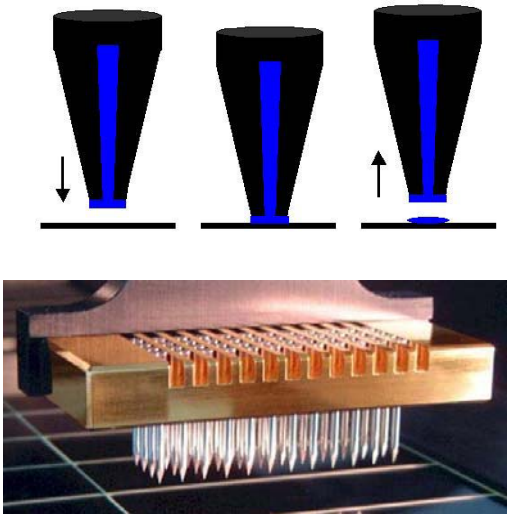


Figure 3.1 Printing process

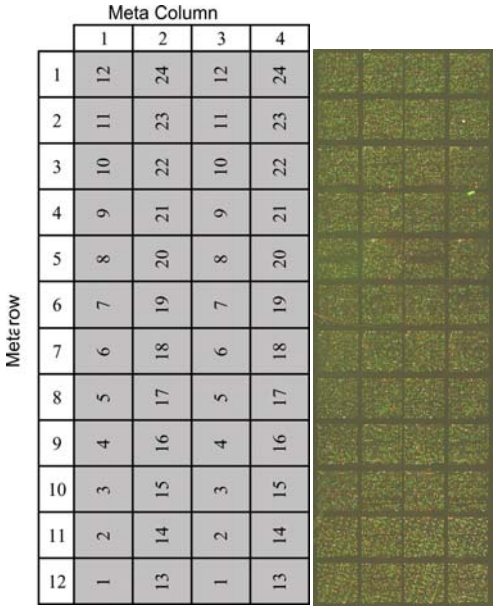


Figure 3.2 The design of 48 blocks

3.2 Experimental design

We used a Cy3 and Cy5 two-color microarray system and dye swap experiments (Figure 3.3). For statistical analysis of data, experiments of each time point were carried out with at least three biological repeats. 200-800 embryos were exposed to different toxicants at different stages and mRNAs were labeled to hybridize two chips.

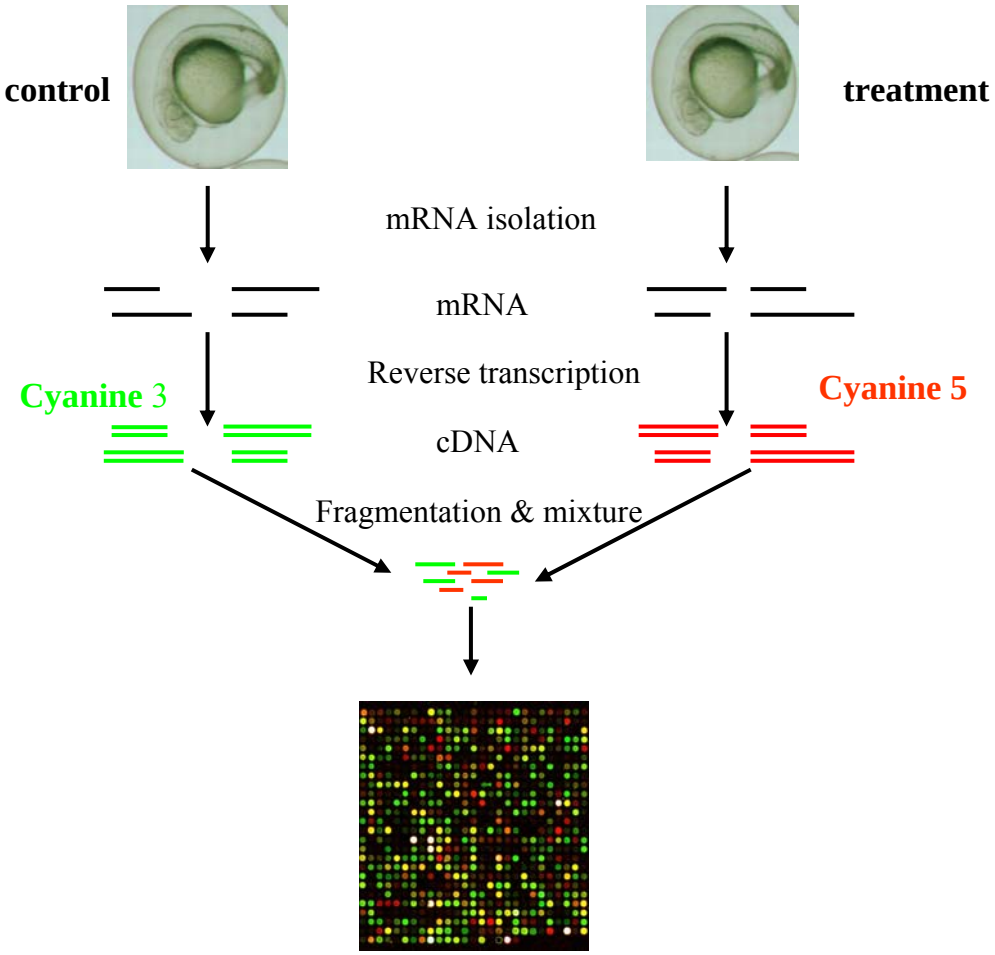


Figure 3.3 Schematics of two colors microarray

3.3 Processing raw data

Arrays were scanned using the Axon model 4000B dual-laser scanner (Molecular Devices) and the corresponding GenePix 6 software (Union City, CA). Both channels

(532 nm for Cy3 and 635 nm for Cy5) were scanned in parallel and stored as 16-bit TIFF files.

The TIFF images were processed by the GenePix software. After opening the TIFF image and loading the array list file, the spots were overlaid by adjusted grids. The image information can be extracted from every spot pixels and transferred to a specific file with additional parameters, such as foreground and background pixels, Cy5 (532 nm) and Cy3 (635 nm) intensity. The pixels of the background, in principal, are supposed to be zero. Nonetheless, background fluorescence is observed because of non-specific binding and the problems in postprocessing. Background estimation was applied to calculate averaging of all pixel intensities except the hybridization spot intensities. We also used a local background estimation to compensate background noise (Figure 3.4).

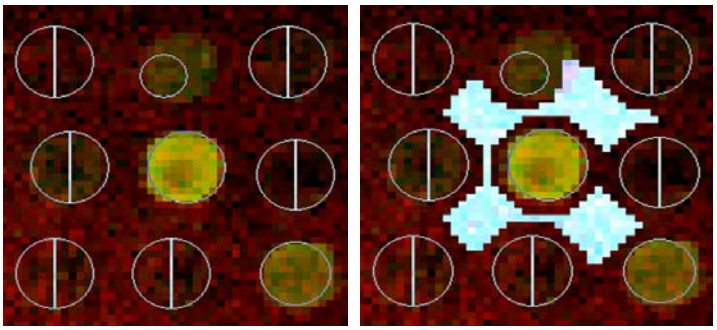


Figure 3.4 Background pixels

3.4 Data transformation and normalization

Raw data must be preprocessed before statistical analysis. In particular, preprocessing consists of transformation (appropriate scaling) and normalization (systematic error correction). The fluorescence intensities of spots are transformed using logarithmic scale (Figure 3.5). Differences in gene expression of raw data are represented by the Fold Change y_{red}/y_{green} (y means spot intensity). On the contrary, changes on logarithmic scale are calculated as the difference between channels, $\log(y_{red})-\log(y_{green})$. Systematic multiplicative error terms become additive and are automatically eliminated by subtracting the channel intensities. For the normalization, we used MA-plots. M is the log

ratio of raw intensities and means log(y_{red})-log(y_{green}). A represents the average expression level of the spot and means log(y_{red}+y_{green})/2.

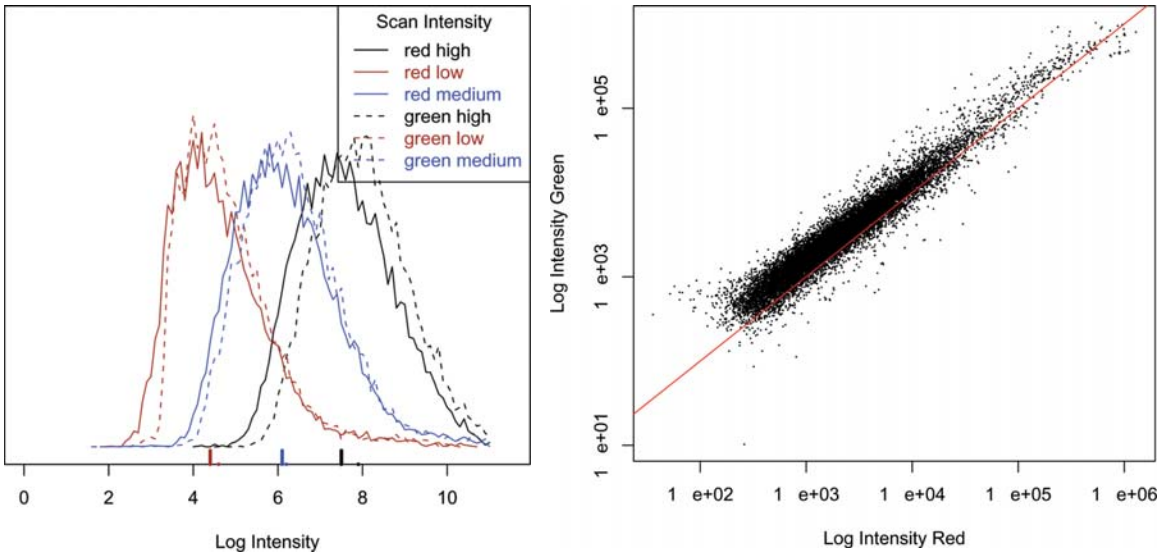


Figure 3.5 Distribution of intensities

3.5 Microarray quality control

3.5.1 Quality control on spot level

In the processing of microarrays, abberations, like scratches, air bubbles and printing problems can induce influences and bias. In order to get available data, these erroneous signals have to be detected. If these artefacts can not be compensated, these spots or even the whole chip has to be discarded. We set up criteria to control the microarray quality.

A normal diameter of a spot is 100 µm and stretches over 10 pixels after scanning with a resolution of 10 µm. If the diameter of a spot was less than 70 µm or more than 140 µm, it was excluded in the subsequent processing. The fluorescence intensities of spots are presented as foreground signals. If the foreground intensities of a spot was less than 1.75 times intensity of its background, it was discarded. For the inconsistent spots presented by printing, we used the coefficient of variation (CV) to filter some defective spots.

$$CV_k = \frac{\text{Standard Deviation of Pixels in Spot } k}{\text{Mean Intensity of Pixels in Spot } k}$$

If the coefficient of variation of a spot is more than 0.7, it was filtered. Additionally, a spot was discarded if more than 20% of its pixels are in saturation, i.e. its signals exceed the maximum 16-bit intensity value of 65535 (2^{16}).

3.5.2 Quality control on array level

For the whole chip, plotting the distribution of *M* values was used to evaluate the quality of the microarray. Basically, if the microarray has a good quality, the *M* values cluster around 0 based on the assumption that the majority of genes is not differentially regulated under toxic conditions (Figure 3.6). The majority of microarray experiments revealed a very prominent intensity dependent signal pattern mainly due to different physicochemical traits of the two fluorescence dyes. Application of a locally weighted regression smoother (LOESS) successfully suppressed this effect and was hence applied to the log-transformed data.

3.5.2.1 Covariance

The quality control measures evaluate the single chip but can also be used to compare different microarrays. Covariance measures how much paired signals of spots in two chips vary between each other. The more signal pairs differ from the mean values in the same direction, the more positive becomes the covariance.

3.5.2.2 Correlation

Scatter plots and statistical parameters are used to determine the correlation between chips. Visual correlation analysis is performed for the log transformed average intensities *A*, which is log $(y_{red}+y_{green})/2$ and for the log transformed differences *M*, which is log y_{red}- log y_{green}. Basically, the perfect correlation of average intensities *A* would be in an identity line with a 45° slope. *M* values are subdivided into two groups. The first group

includes genes with an adjusted p-value >0.05, which are considered to be genes whose expression is not altered. The second group includes the genes with an adjusted p-value ≤0.05, which can be considered as the genes whose expression were changed by the manipulations.

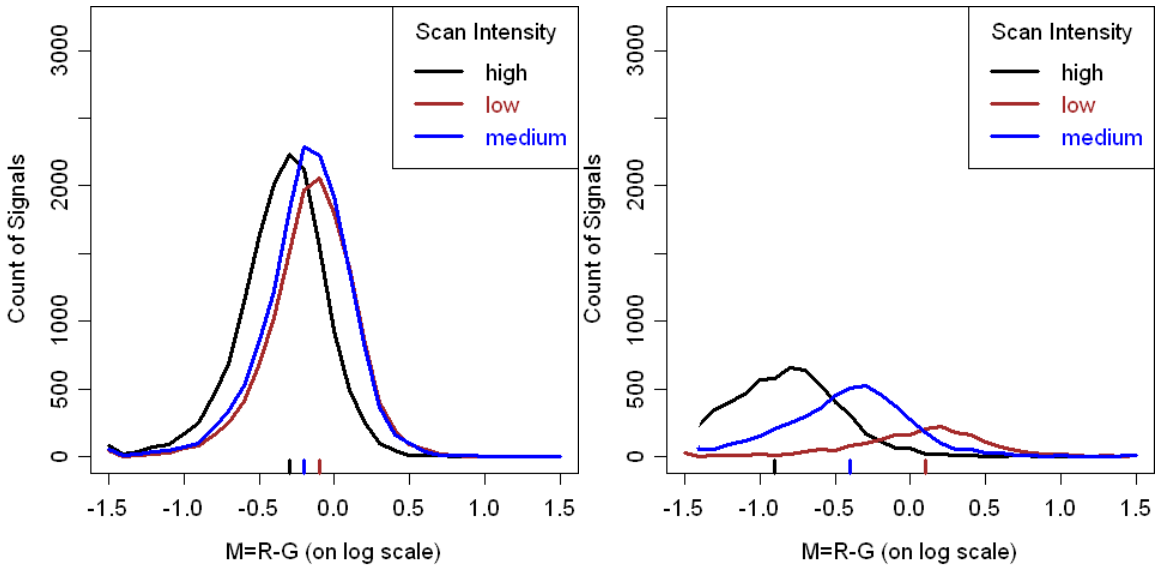


Figure 3.6 Distribution of ***M***

3.6 Statistical tests

In order to get more confidence into the data, we carried out statistical tests that relate the changes in gene expression to variability.

3.6.1 t-test

The t-test is applied to relate the signal intensities to their variations and the t-test poses three major assumptions: normal distribution, homoscedasticity, and sufficient sample size. Because of the small sample size from each gene, we modified the t-test methods accordingly and weighted gene specific standard error and global standard error among all genes.

3.6.2 Multiplicity

The number of genes in the zebrafish chip is more than 10000. If a statistical test for B genes is carried out with α=0.05, the probability of making one false positive is $1-(1-\alpha)^B$. It means that we will have 500 false positive results. In order to reduce these false positive probabilities, we adjust the p-values for multiple testing. Adjustment is based on False Discovery Rate (FDR) which is the expected proportion of False Positives among all positive findings.

3.7 Gene selection

To take into account the extent and significance of gene expression as well as different or similar expression between all toxicants, we consider a consensus selection based on adjusted p value, fold change, MAD (median absolute deviation) and Pearson correlation to be the filters. As a result, 150 to 300 genes that were significant at an adjusted significance level of α equal to 0.025 were picked up for further statistical clustering and classification.

3.8 Principal component analysis

Principal component analysis is a statistical framework to exploit information from large datasets of high complexity by highlighting and extracting underlying patterns. We used this method to analyse gene expression profiles under different conditions, such as different toxicant exposures, different timepoints and different toxicant concentrations. A simple condition is an example to introduce the principle of this method.
The example is based on a dataset comprising two corresponding variables x and y which represent the expression data for two treatments.

x_{raw}	2.5	0.5	2.2	1.9	3.1	2.3	2.0	1.0	1.5	1.1	1.0	2.5
y_{raw}	1.7	0.5	2.0	1.5	2.1	1.9	1.1	0.8	1.1	0.6	2.2	0.5

Working with two dimensions allows visualization of the PCA algorithm. However, the method works accordingly with any number of dimensions T (treatments) × G (genes).
The first step in PCA is to centre each variable by subtracting the means (x = 1.8 and y = 1.34) from both treatment data.

x	0.7	-1.3	0.4	0.1	1.3	0.5	0.2	-0.8	-0.3	-0.7	-0.8	0.7
y	0.4	-0.8	0.7	0.2	0.8	0.6	-0.2	-0.5	-0.2	-0.7	0.9	-0.8

Secondly, the Covariance Matrix is calculated. It provides the observed variances between and among centred variables x and y, thus

$$\mathbf{C} = \begin{pmatrix} \mathrm{cov}(x,x) & \mathrm{cov}(x,y) \\ \mathrm{cov}(y,x) & \mathrm{cov}(y,y) \end{pmatrix} = \begin{pmatrix} 0.61 & 0.23 \\ 0.23 & 0.43 \end{pmatrix}$$

The third step, the Eigenvectors and Eigenvalues are derived for the Covariance matrix. A square matrix, like the Covariance matrix C, with T ×T dimensions has T Eigenvectors EV such that

$$C \cdot EV = \lambda \cdot EV$$

Here, λ are the corresponding Eigenvalues. Hence, if an Eigenvector is transformed by the matrix C it results in a multiple of itself because the transformation only changes the length of the vector but do not change its direction. The Eigenvalue is the amount by which the original vector is scaled after multiplication by the square matrix C. The parameters for the covariance matrix C in the example are calculated as

$$\mathbf{EV_1} = \begin{pmatrix} 0.83 \\ 0.56 \end{pmatrix} \quad \text{with } \lambda_1 = 0.77$$

$$\mathbf{EV_2} = \begin{pmatrix} 0.56 \\ -0.83 \end{pmatrix} \quad \text{with } \lambda_2 = 0.27$$

The Eigenvector with the largest Eigenvalue, EV_1 is the first principal component of this data. It explains the greatest amount of variance. EV_2 is perpendicular to EV_1 and means the remaining variation (Figure 3.7). In the next step, the Eigenvectors are sorted by their importance, i.e. by descending Eigenvalues. All Eigenvectors are perpendicular to each other. Accordingly, the data can be expressed in terms of these Eigenvectors instead of

expressing them in terms of the *x* and *y* axes. Therefore, the expression data is plotted along the Eigenvectors – the principal components (Figure 3.8). The lesser significance components might represent technical noise in microarray data. Therefore, the expression data can be normalized by ignoring Eigenvectors with small Eigenvalues. Hence, the dimension of the data is reduced by multiplying the original data only with a subset of the most important Eigenvectors. The subset of most important principal components can be determined in various ways. For instance, the subset has to explain at least some fixed percentage of variation, or can be picked up by all components whose Eigenvalues is greater than the average of all Eigenvalues.

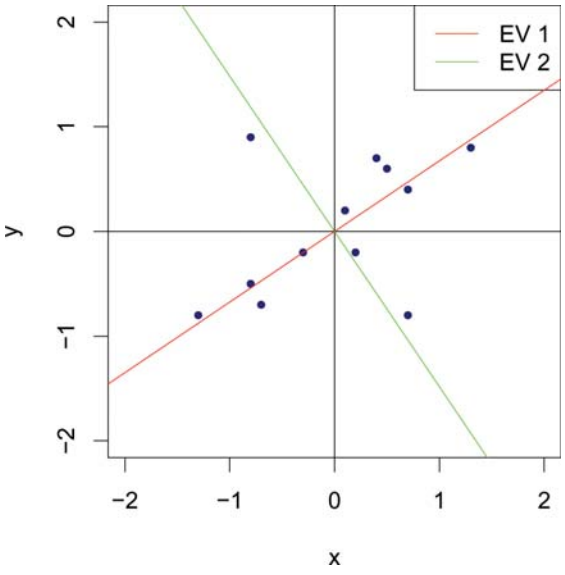


Figure 3.7 Eigenvectors

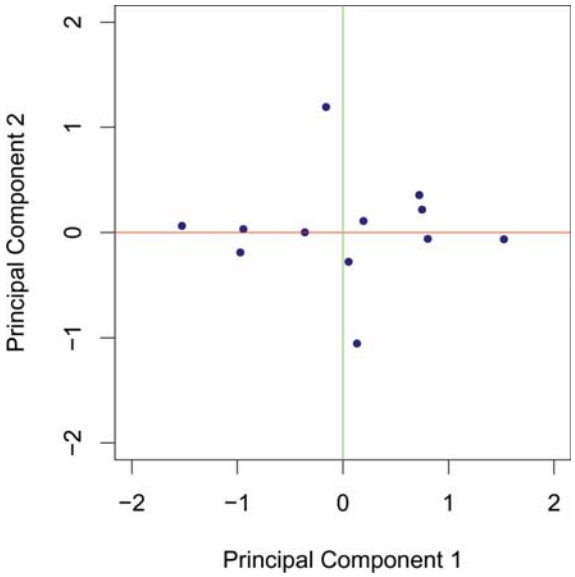


Figure 3.8 Rotation to PC-space

3.9 Cluster analysis

Cluster analysis is an integral part of microarray analysis. Compared with other multivariate statistical techniques, it extracts and summarizes information out of expression profile that is available for thousands of genes under various conditions. The basic objective of cluster analysis is to split data into different clusters that have similar objects. Thus, elements within the same cluster are more similar to each other than that in another cluster.

3.9.1 Proximity measures

Clustering is based on proximity measures that quantify how far two observations are from each other. Proximity can be either a similarity measure or a dissimilarity measure. The most commonly used dissimilarity measure is the Euclidean distance which defines the geometrical distance of x_i and y_i in a space of n-dimensions.

$$d_{Eucl} = \sqrt{\sum_{i=1}^{n} (x_i - y_i)^2}$$

We applied the Pearson Correlation as proximity measure to tackle the complex biological situation.

$$d_{Pear} = \frac{\sum_{i=1}^{n} (x_i - \bar{x}) \cdot (y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n} (x_i - \bar{x})^2} \cdot \sqrt{\sum_{i=1}^{n} (y_i - \bar{y})^2}}$$

We applied also the Arcus Cosinus function to the distance measure. Besides transforming correlation to a dissimilarity measure, this function decreases the distance between a pair of highly correlated genes. Subsequently, the relationship between two very similar genes is attributed an even higher importance in forthcoming clustering steps.

$$d_{unidir} = \arccos(d_{Pear})$$

$$d_{bidir} = \arccos(|d_{Pear}|)$$

3.9.2 Hierarchical clustering

Hierarchical clustering consists of agglomerative and divisive hierarchical clustering. Agglomerative hierarchical clustering is started by assigning each of the n observations an own cluster of size 1. The pair of observations x_i and y_i, which have the smallest distance from each other is picked up and merged into a common cluster. And then, the distance matrix is recalculated in order to take into account the distance of the new

cluster to the other observations. The sequence of identifying the two clusters with the highest similarity, their agglomeration, and the recalculation of the distance matrix is repeated until all observations aggregate in one single cluster.

On the other hand, divisive hierarchical clustering is initiated by assigning all observations to the same cluster. And then, it is split in the two subsets with the biggest distance to each other.

4. Results

4.1 The choice of exposure windows

Currently, hazard assessment of chemicals for fish is based on international standards (ISO, ASTM) and guidelines (OECD) based on global toxicity endpoints such as mortality or impairment of growth and reproduction. Under increasing pressure of animal welfare groups, the chemical industry searches for alternatives to reduce the number of used organism. A significant step forward is the willingness of the ecotoxicologist community and of the pharmaceutical industry to apply the principles of replacement, reduction, and refinement in the context of regulatory environmental assessments (Hutchinson et al., 2003). One of the alternatives proposed is to use the early life stages of fish as an experimental model. In fact, the early life stage of zebrafish has become a standardized assay for sewage testing in Germany replacing traditional tests with adult fish (Braunbeck et al., 2005).

The biological response to toxicants can vary at different life stages. It is largely accepted that vertebrates at the earliest life stages are more responsive to chemical insult (Grandjean et al., 2006). Evidence has been accumulating over several decades that industrial chemicals can cause neurodevelopmental damage and that subclinical stages of these disorders might be common (Grandjiean et al., 2006). The toxic effect is assessed through the analysis of various criteria: mortality, hatching (rate, time), and larvae morphology (length, weight, abnormality). The DIN standard relies on the embryonic stage up to 48 hpf and the selected endpoints refer to rather crude defects in embryogenesis such as somite development, tail detachment, heart beat, and coagulated eggs. The guidelines did, however, not provide good reference information about which stages are best suited to study gene expression profiles. An exposure for 48 hpf may also induce secondary effects that are not directly related to a particular chemical and may also be more variable from animal to animal. Also, stages may differ in their usefulness for evaluating gene expression profiles for classification of the toxicogenomic effect of different chemicals. To tackle this question, I have chosen to expose zebrafish embryos at

three different developmental stages. Moreover, I limited exposure to a period of 20 to 24 hours. The 4 to 24 hpf treatment covers late blastula, gastrula and segmentation stages during which the overall body plan is laid down. The treatment phase between 24 to 48 hpf coincides with organogenesis. The first neuronal circuits become active and touch-evoked movements can be observed from this stage onwards. During the 96 to 120 hpf period, organogenesis has proceeded so much that the animal will be ready to feed. We expect that many physiological responses that also characterize the adult zebrafish are active by this stage.

I next had to decide which concentration to use. To this end, I determined the concentrations that would induce in approximately 50% embryos morphologically visible effects. At the same time I had to keep the concentration so low that I could minimize lethality or apoptosis/necrosis in the treated embryos. I took different exposure windows into account and scored morphological changes using the criteria below: The embryos that had been exposed to toxicants either from 4 to 24 hpf or 24 to 48 hpf or 96 to 120 hpf were washed twice in fresh fish medium and cultured in the incubator (28.5°C) to 72 hpf and 144 hpf (from 96 to 120 hpf) respectively. Abnormal larvae were counted and the percent morphological defects were calculated. Accordingly, concentrations of chemicals were used which could cause approximately a defect in 50% of embryos. This is just an approximation as the effective concentration varied with respect to different morphological endpoints (Table 1 and 2).

Table 1 Percentage of morphological defects at 72 hpf of 4 to 24 hpf exposure group

	Head %	Trunk %	Yolk sac %	Edema %
4CA (15 mg/l)		66.1	67.3	53.8
DDT (5 mg/l)	27.3	63.8	68.1	25.4
TCDD (150 ng/l)	68.2	89.3	87.4	83.2
VA (15 mg/l)	10.1	62.7	40.6	55.3
MeHg (50 µg/l)	72.4	67.9	61.5	63
Cd (500 µg/l)	5.3	57.6	10	7.2
Control (embryo water)	-	1.3	-	1
VC1 (0.2% ethanol)	-	1.8	1	1
VC2 (28 mg/l toluene, 0.5% DMSO)	-	1.5	2	1.2

Table 2 Percentage of morphological defects at 72 hpf of 24 to 48 exposure group

	Head %	Trunk %	Yolk sac %	Edema %
4CA (50 mg/l)	-	68	69.2	55.6
DDT (15 mg/l)	30.2	65.1	57.3	28.4
TCDD (500 ng/l)	73.1	91.9	78.8	80.5
VA (50 mg/l)	-	65.3	43.1	55.8
MeHg (60 µg/l)	75.1	68.3	46.8	51.3
Cd (5 mg/l)	2.3	62.2	11.1	9.4
Control (embryo water)	-	1.3	-	1
VC1 (0.2% ethanol)	-	1.8	1	1
VC2 (28 mg/l toluene, 0.5% DMSO)	-	1.5	2	1.2

Several hundred embryos were exposed to the 6 model compounds, TCDD; VA; MeHg; Cd; 4CA; and DDT at 4 to 24 hpf, 24 to 48 hpf, and 96 to 120 hpf and treated embryos were harvested and mRNAs were extracted. Cy3 and Cy5 labeled cDNAs were prepared and hybridized to the oligonucleotides array. Arrays were scanned with an Axon 4000B scanner. Data were normalized, and averaged from different experiments before they were subject to different clustering methods (see Material and methods).

4.1.1 Principal component analysis

Principal component analysis is a statistical framework to exploit information from large datasets of high complexity by highlighting and extracting underlying patterns and is often applied to present grouping structure of clustering results. Principal component analysis of the toxicogenomic profiles of embryos exposed to the 6 compounds from 4 to 24 hours showed that similar expression profiles were induced. The profiles clustered closely together in the 2 dimensional plot shown in Fig. 4.1. On the other hand, the scatter plots of embryos treated from 24 to 48 hpf and from 96 to 120 hpf showed large distances between the toxicogenomic profiles of the six different chemicals. This indicates that the expression profiles are characteristic for each of the 6 compounds at these two stages (Fig. 4.1).

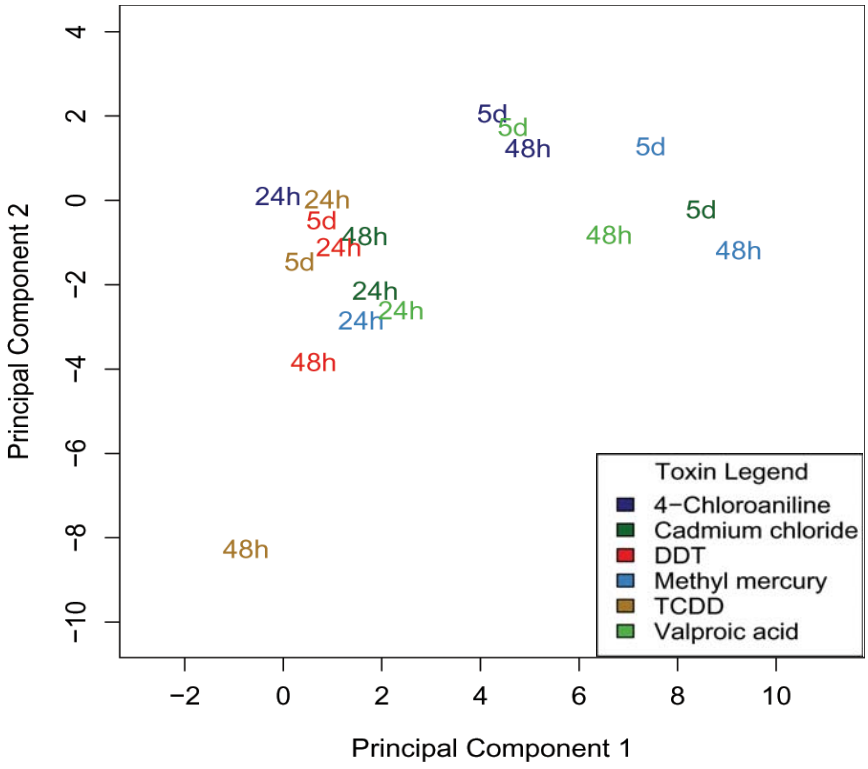


Figure 4.1 Principal component analysis of the toxicogenomic profiles derived from 3 different embryonic stages. Embryos were exposed to 6 chemicals: TCDD: 150 ng/l (24 hpf), 500 ng/l (48 hpf), 500 ng/l (120 hpf); MeHg: 50 µg/l (24 hpf), 60 µg/l (48 hpf), 60 µg/l (120 hpf); VA: 15 mg/l (24 hpf), 50 mg/l (48 hpf), 50 mg/l (120 hpf); 4CA: 15 mg/l (24 hpf), 50 mg/l (48 hpf), 50 mg/l (120 hpf); Cd 500 µg/l (24 hpf), 5 mg/l (48 hpf), 5 mg/l (120 hpf); DDT: 5 mg/l (24 hpf), 15 mg/l (48 hpf), 15 mg/l (120 hpf) either from 4 to 24 hpf or from 24 to 48 hpf or from 96 to 120 hpf. The concentrations were adjusted to that they caused visible morphological effects but little cell or embryo death.

4.1.2 Hierarchical cluster analysis

Common objectives in microarray experiments are gene identification, class discovery, and class prediction. Class discovery usually refers to identifying previously unknown sample subtypes from the study of gene expression profiles. Class prediction is to predict the class membership of a new sample based on a gene expression prediction function. Hierarchical cluster analysis is commonly used as a method for class discovery. Cluster analysis techniques have been applied to organize gene expression data by grouping genes or samples with similar patterns of expression. In clustering samples

overexpression levels of multiple genes, the expression patterns of the samples in the same group are more homogeneous as compared to the expression patterns in the other group. Clustering samples is used to characterize similar/distinct samples or to discover new sample classes. The hierarchical clustering algorithm forms clusters in a hierarchical fashion resulting in a tree-like dendrogram (see microarray data analysis).

The expression profiles summarize clustering results of a subset of 751 genes induced by the six chemicals at the three stages (Figure 4.2). The applied gene selection criteria take into account the extent and significance of changes in gene expression (P_{adj} <0.025 and |FC|>2.0). Distinct toxicogenomic patterns were noted at the three stages. The gene expression patterns were less prominent in the data sets from 4 to 24 hpf treatment groups and only 57 genes were significantly regulated (Figure 4.3). The treatment groups from 24 to 48 hpf and from 96 to 120 hpf induced the most diverse changes in gene expression (Fig. 4.2): 480 and 311 genes were regulated in 24 to 48 hpf and 96 to 120 hpf exposure groups by the 6 toxins, respectively (Figure 4.3). Of these groups, 4CA, MeHg and VA induced related but distinct gene expression patterns between 48 and 120 hpf (Figure 4.2). Based on the gene expression profiles of the embryos exposed to the 6 different chemicals at three stages, using the same criteria (P_{adj} <0.025 and |FC|>2.0) as filters, 6 genes are regulated in all stages; 9 genes are affected in 24 and 48 hpf treatment groups; 4 genes are induced in both the 24 and 120 hpf treatment groups; 68 genes are significantly up or down-regulated in the 48 and 120 hpf treatment groups (Figure 4.3). This shows that there is a strong stage specific effect in responses. Thus, the stage, at which the toxicogenomic response is measured, has to be taken into account.

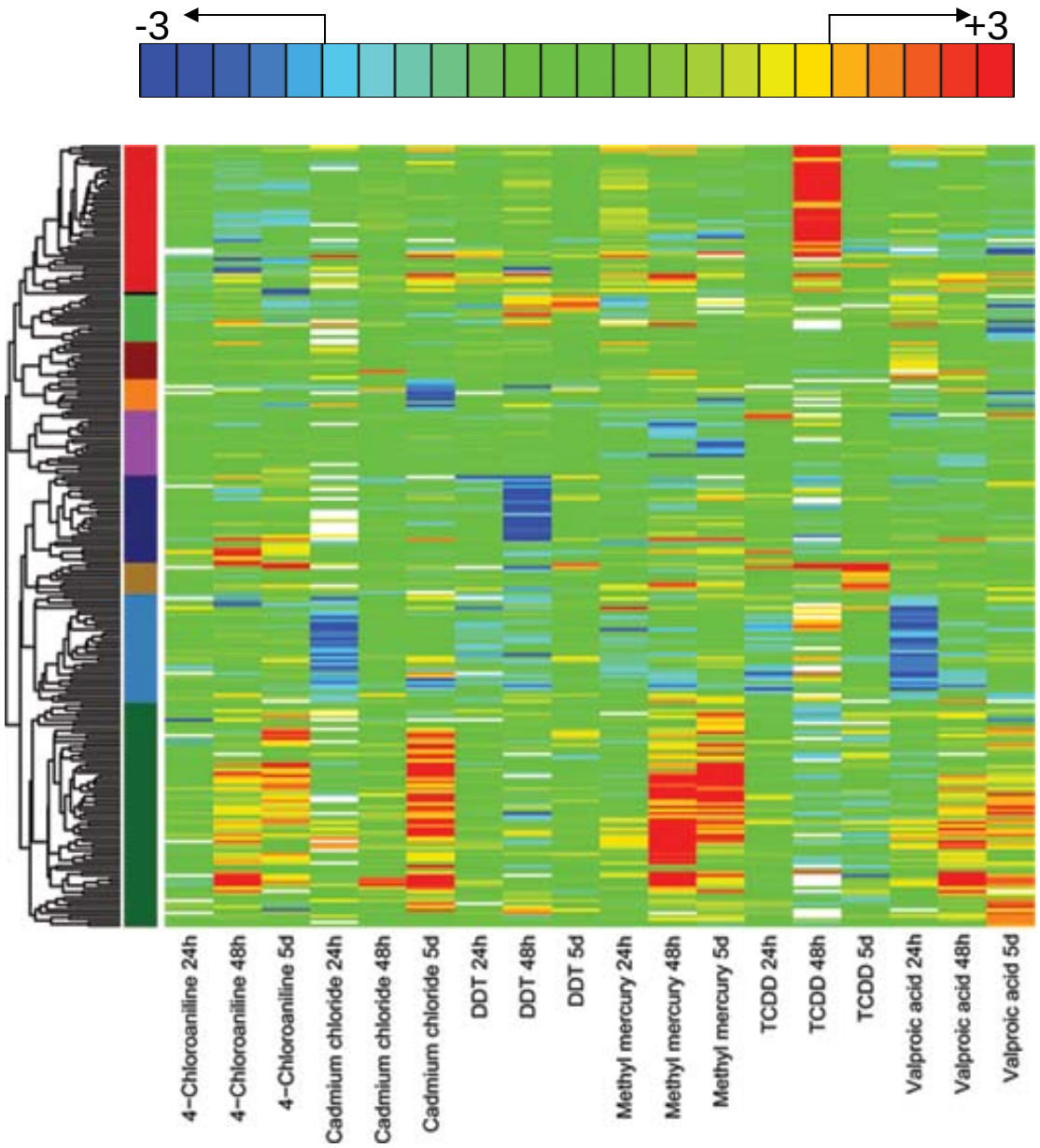


Figure 4.2 Expression profiles at different embryonic stages. Hierarchical clustering of gene responses in embryos treated either from 4 to 24 hpf or from 24 to 48 hpf or from 96 to 120 hpf with 4CA: 15 mg/l (24 hpf), 50 mg/l (48 hpf), 50 mg/l (120 hpf); Cd: 500 µg/l (24 hpf), 5 mg/l (48 hpf), 5 mg/l (120 hpf); DDT: 5 mg/l (24 hpf), 15 mg/l (48 hpf), 15 mg/l (120 hpf)); MeHg: 50 µg/l (24 hpf), 60 µg/l (48 hpf), 60 µg/l (120 hpf); TCDD: 150 ng/l (24 hpf), 500 ng/l (48 hpf), 500 ng/l (120 hpf); VA: 15 mg/l (24 hpf), 50 mg/l (48 hpf), 50 mg/l (120 hpf). The colour code indicates fold-changes ranging from 3-fold up- (red) to 3-fold down-regulated (blue). Fold changes larger than 3-fold are not indicated explicitly but are subsumed as 3-fold up- or down-regulated. Genes were listed whose mRNA levels changed by more than 2-fold (adjusted $p<0.025$) in at least one of the treatments.

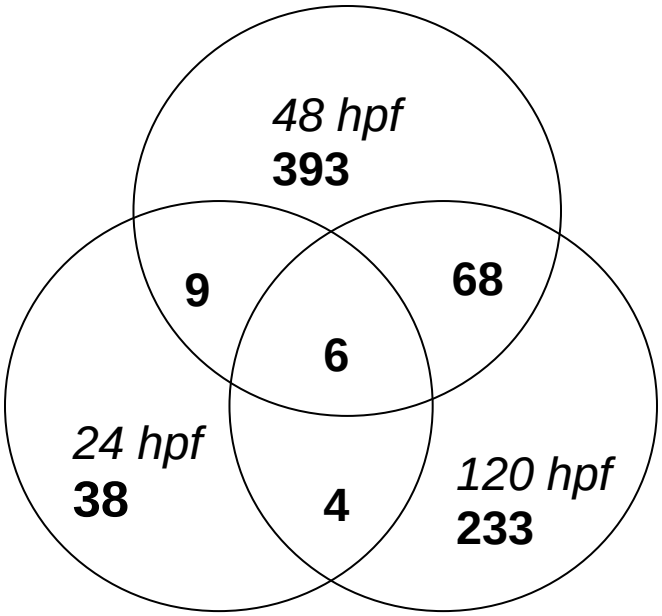


Figure 4.3 Number of genes regulated by the six model chemicals at the different time-points. The numbers in the different intersections indicate the number of genes that were induced at either one or two or all three stages. The criteria used to select genes are P_{adj} <0.025 and |FC|>2.0.

4.2 96-120 hpf exposure study

Based on the comparison of the three exposure windows, there are most significant changes in gene expression and explicit classification of chemicals in treated embryos from 24 to 48 hpf and from 96 to 120 hpf. Compared with embryos treated from 24 to 48 hpf, the exposure stage from 96 to 120 hpf has several advantages: At 96 hpf, the hatched larvae have completed most of the early morphogenesis and begin to swim. They also start to feed and hunt on the subsequent stages suggesting that they have an almost mature physiology and may thus have also more adult responses to toxicants. Experimentally, these older stages have the advantage that one needs to expose fewer animals to isolate sufficient RNA for a microarray experiment. For these advantages of the 96 to 120 hpf exposure window I focused the systematic analysis of toxicogenomics profiles on this stage and expanded also the repertoire of toxicants by including additional 5 toxicants (Pb, As, tBHQ, Acrylamide, PCB (Aroclor 1254)).

To further evaluate the significance of the signature genes in relation to the different chemicals, the embryos were exposed to 11 different toxicants from 96 to 120 hpf and the gene expression profiles were analysed by different statistical methods. Experiments were repeated on a technical and biological level. Replicate hybridizations with mRNA from at least three independent toxicant exposures were performed.

4.2.1 Analysis of gene expression profiles of 96-120 hpf exposure

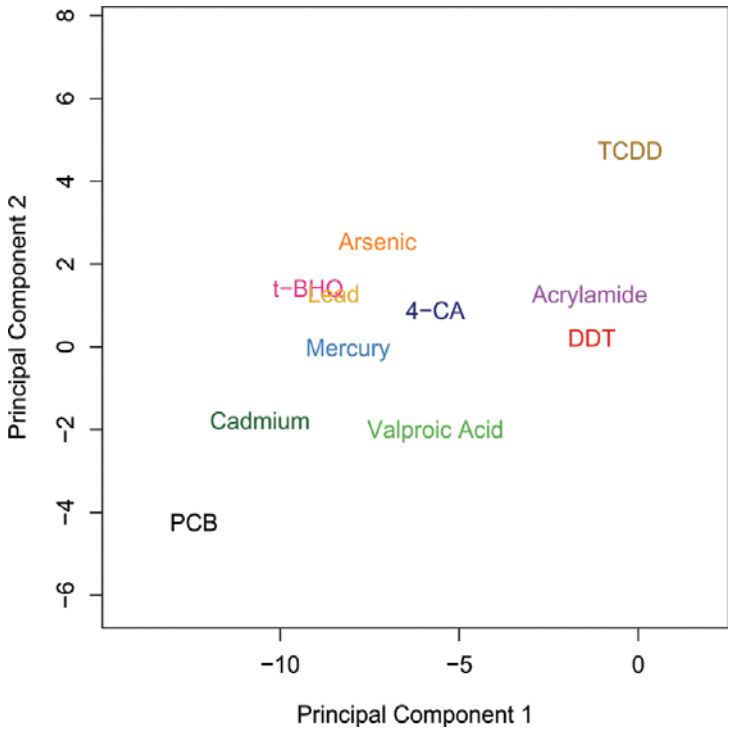


Figure 4.4 Principal components analysis of 96-120 hpf treatments. Principal component analysis showing the similarity and differences in gene response in embryos treated between 96 and 120 hpf with the PCB (33 mg/l); TCDD (500 ng/l); 4-CA (50 mg/l); DDT (15 mg/l); Acrylamide (71 mg/l); Arsenic (79 mg/l); Lead (2.8 mg/l); Cadmium (5 mg/l); t-BHQ (1.7 mg/l); Mercury (60 µg/l); Valproic Acid (50 mg/l). The 11 clusters are separated from each other according to their Euclidean distances.

The principal component analysis reveals that the toxicogenomic responses of the 11 different toxicants are scattered in different positions. The positions induced by t-BHQ and Pb are very close in two dimensions (Figure 4.4). However they are separated in 3 dimensions. In other words, the gene expression profiles in embryos treated with the 11

different chemicals are characteristic for every chemical suggesting different model of toxic action.

The expression profiles (Fig. 4.5) summarize clustering results of a subset of 199 genes across all 11 toxin responses. Distinct patterns of gene expression were found for each of the 11 compounds. However, similarities in the gene responses were also observed. One group of chemicals with related gene responses includes Pb, As, Cd, tBHQ, MeHg and VA. Another subgroup of related responses was induced by TCDD, 4CA, DDT and AA, whereas the PCB triggered a more distinct expression profile.

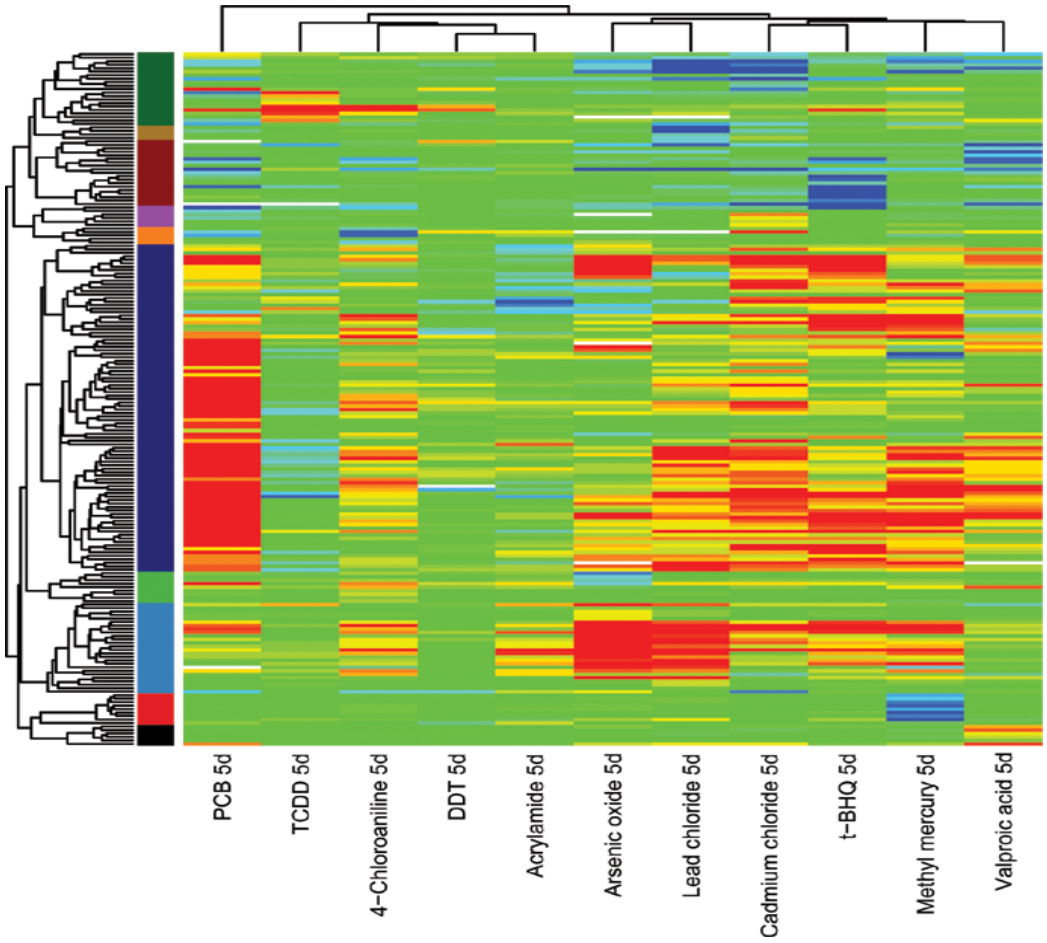


Figure 4.5 Hierarchical clustering of gene responses in embryos treated between 96 to 120 hpf. PCB: 33 mg/l; TCDD: 500 ng/l; 4-CA: 50 mg/l; DDT: 15 mg/l; AA: 71 mg/l; As: 79 mg/l; Pb: 2.8 mg/l; Cd: 5 mg/l; tBHQ: 1.7 mg/l; MeHg: 60 µg/l; VA: 50 mg/l. The colour code indicates fold-changes ranging from 3-fold up- (red) to 3-fold down-regulated (blue). Fold-changes larger than 3-fold are not indicated explicitly but are subsumed as 3-fold up- or down-regulated.

4.2.2 Selected response genes

Gene name	Gene ID	AA	As	4CA	Cd	DDT	Hg	Pb	PCB	tBHQ	tcdd	VA
Peroxiredoxin 1	BI980610	3.9	13.6	4.1	3.1		7.7	9.9		7.5		
Thioredoxin	BI864190		14.2	3.5	4.0		4.4	8.1	2.5	6.1		
Stress protein HSP70,	AB062116		10.1	2.2	7.0			2.5	5.4	12.3		2.7
Activating transcription factor 3	AW422298		4.0		3.2		3.3	2.5	15.1	5.1		5.4
Zinc fingers and homeoboxes 1	AI793802		2.5		2.6		2.7	2.4	4.2	2.1		2.0
Sequestosome 1	AW343560		10.3	2.1	3.9		3.2	3.5	2.8	5.1		
unknown	BG985532	2.6	8.8	2.3				4.3	2.6	2.9		
Glutathione S-transferase omega1	AW019036	3.0	6.3	2.1			2.6	3.5		2.2		
matrix metalloproteinase 9	AW174507		2.1		12.3		6.7	3.9	14.2	6.4		
Hsp70	AF210640		10.9		6.6			2.5	5.0	10.8		2.7
6-phosphogluconate dehydrogenase	AW115782		3.3	2.0			2.2	3.4	2.0	2.1		
Likely hypoxia induced gene 1	BI892416		2.6		2.7		2.3	3.4	2.7	2.2		
Fetuin-A,	AI496863		-3.6	-2.2	-3.3			-3.2	-5.0			-2.3
unknown	AW115990		3.1				3.2	2.3	5.6	4.6		3.1
Hsp70	AF006007		7.6		6.3			2.1	4.6	10.1		2.4
matrix metalloproteinase 13	AW305943			2.5	11.9		5.9	3.5	15.1	10.2		
Angiotensinogen precursor,	BG727310			3.0	2.7		3.0	2.1	3.7	2.1		
Solute carrier family 16,me 9	BE016639			2.5	2.9		2.4	3.6	4.1			2.3
Solute carrier family 16,me 9	BI474827			3.4	4.3		4.8	4.7	7.9			3.0
GSH S-transferase pi	AF285098	2.0	4.1				3.2	5.9		2.7		
CCAAT/enhancer binding protein, beta	AW019436		2.1		2.3			2.4	6.9	2.3		
unknown	AW117109		2.3				2.1	2.1	2.3	3.2		
unknown	AI964296		2.1		2.2		2.1	2.1	2.0			
Syncollin	AA566708		-2.1		-2.7		-2.3	-3.8				-2.2
Cofilin 2 (muscle)	BI883993			2.4			3.3	3.6	3.1			2.2
unknown	BG727211			3.4	2.3		3.1		2.4	2.7		
Serum/glucocorticoid regulated kinase 2	BI673466			2.0			2.6	2.2	3.6			2.0
Cytochrome P450 1 A 1	AF057713			3.7		2.5			4.2	2.8	31.6	
unknown	AW019018			3.6	2.8		4.9		2.5	5.9		
Suppressor of cytokine signaling3	BG727181			2.0	2.5		4.5		5.6	3.7		
Complement component 7	AA497156				7.4		3.4	6.2	7.3	3.7		
ADP ribosylation factor 1	AI477644				3.5	-2.2	12.4		46.3			8.0
Gastric intrinsic factor)	AI353694	2.2	4.6					2.5			2.1	
Multidrug resistance-associated protein Mrp2,	AW279805		2.6	2.4			2.4	2.3				
Arsenite inducible RNA associated protein,	BM182280		5.1				2.4	2.5		2.7		
GSHS-transferase omega 2	BI979918		5.6		2.7			5.2		2.1		
Pvalb3a	BE201681		-2.4		-4.3		-2.6	-5.1				

Table 3 Comparison of selected genes expression changes induced by the 11 chemicals. The numbers are representative up- or down-regulated genes in response to

exposure to the 11 chemicals. Blanks indicate no significant difference in gene expression compared with the control conditions using filters with P_{adj} <0.025 and |FC|>2.0. The gene name is that of the zebrafish gene or of the closest human homologue. The gene ID is indicated by the GeneBank accession number. In this table, only genes that were regulated by at least 4 chemicals were included. The complete list is provided in the appendix.

4.2.2.1 Activating transcription factor 3

Activating transcription factor 3 (*ATF3*) gene, which encodes a member of the activating transcription factor/cAMP responsive element binding protein (ATF/CREB) family of transcription factors, is maintained at a low level in quiescent cells. However, ATF3 is rapidly induced by a wide-range of stresses including post-seizure brain, nerve axotomy and hepatotoxicity. In human, the 5'-flanking region of *ATF3* has a number of transcription factor binding sites including the AP-1, ATF/CRE, NF-κB, E2F, and Myc/Max binding sites. Previous studies revealed that the level of *ATF3* mRNA increases greatly in tissue culture cells after serum stimulation. Studies in the rat showed that the *ATF3* mRNA level increased in mechanically injured liver after partial hepatectomy and in chemically injured liver treated with toxins such as carbon tetrachloride or alcohol (Liang et al., 1995). Overexpression of ATF3 prevents p53 from MDM2-mediated degradation and leads to increased transcription from p53 regulated promoters (Yan et al., 2005). According to the gene expression profiles of 96-120 hpf exposure embryos, *ATF3* mRNA was up-regulated significantly by As, Cd, MeHg, Pb, PCB, tBHQ, and VA treatment (Table 3) suggesting that the zebrafish embryo responds in a similar way as rat hepatocytos.

4.2.2.2 Antioxidant defence

There are two parallel, yet interdependent enzymatic systems that combat oxidative damage in humans: one anti-oxidant system is based on glutathione as a reducing substrate, and the other system uses thioredoxin as a sulfur-based reductant. The glutathione system is more effective in reducing small disulfide molecules and in reacting

directly with reactive oxygen species (ROS), whereas thioredoxin is more effective in reducing the exposed disulfides of proteins (Winyard et al., 2005). Reduced glutathione (GSH) exists in equilibrium with its disulfide form (GSSG), and the ratio of GSH to GSSG could be used as an indicator of the redox status of the cell. GSH-dependent defence relies on the availability of GSH, and systems involved in the reduction of GSSG are essential. Thioredoxin is regarded as an important oxysensor within cells, whereby its sensing ability is mostly the result of its cysteine redox chemistry and its ability to react with a wide range of different proteins. Thioredoxin has the capacity to repair oxidized proteins by reducing protein disulfides at the expense of the oxidation of its own redox-active cysteine residues. The redox-active cysteine residues are reduced by thioredoxin reductase.

4.2.3 Genes grouped according to gene ontology

4.2.3.1 Glutatione and thioredoxin

Table 4A Glutathione and thioredoxin system

Gene name	Gene ID	AA	As	4CA	Cd	Hg	Pb	PCB	tBHQ	VA
Peroxiredoxin 1	BI980610	3.9	13.6	4.1	3.1	7.7	9.9		7.5	
Thioredoxin	BI864190		14.2	3.5	4.0	4.4	8.1	2.5	6.1	
Glutathione S-transferase omega 1	AW019036	3.0	6.3	2.1		2.6	3.5		2.2	
Glutathione S-transferase pi	AF285098	2.0	4.1			3.2	5.9		2.7	
Glutathione S-transferase omega 2	BI979918		5.6		2.7		5.2		2.1	
Thioredoxin interacting protein	BI892352				2.0					2.0
glutathione peroxidase	AW232474					-4.2				

Peroxiredoxin 1, thioredoxin, glutathione S-transferase omega 1 and 2, and glutathione S-transferase pi were up regulated by 9 chemicals (Table 4A). It is clear that many antioxidant genes were up-regulated in embryos exposed to different toxicants, surprisingly, these antioxidant genes were not regulated significantly by DDT- and TCDD-exposed embryos. In addition, in contrast to thioredoxin, the gluthathion system shows a very versatile response to different toxicants.

The induction of antioxidant gene expression by different chemicals administrated at 96-120 hpf revealed that the genes of the glutathione and thioredoxin systems were triggered significantly by 7 out of 11 toxins. Especially, the four different heavy metals and tBHQ induced a response of these antioxidant pathway genes. Surprisingly, glutathione peroxidase (AW232474) was down regulated robustly by MeHg-exposed embryos (Table 4A).

4.2.3.2 Transporter

Table 4B Transporter and related genes

Gene name	Gene ID	As	4CA	Cd	Hg	Pb	PCB	VA
Solute carrier family 16 member 9 (1)	BE016639		2.5	2.9	2.4	3.6	4.1	2.3
Solute carrier family 16 member 9 (2)	BI474827		3.4	4.3	4.8	4.7	7.9	3.0
Solute carrier family 16 member 6	AW421040				2.9	4.0	4.6	2.9
Solute carrier family 2 member 5	AI477656	2.1				2.1	2.5	
Solute carrier family 6 member 8	BI980828			2.0		2.2	2.4	
Solute carrier family 43, member 2	BI887324						2.1	
Solute carrier family 3	BG985518						2.1	
Solute carrier family 20 (phosphate)member 1	BI890772						2.2	
Solute carrier family 6 (GABA), member 1	BF157011							-3.1
	BI563084							-2.1

Solute carrier family 6 (SLC6) consists of the transmembrane transporters for neurotansmitters, and SLC6 members are thus of fundamental importance for proper signaling between neurons. These transport processes are mediated by distinct classes of membrane transport protein that have key roles in controlling the neurotransmitter concentration in the synaptic cleft. These transporters can be classed as intracellular vesicular transporters that are responsible for sequestering transmitters from the cytoplasm into synaptic vesicles, and plasma membrane transporters that are responsible for sequestering released transmitter from the extracellular space.

SLC6 is a family of plasma membrane Na^+-Cl^- coupled transporter, which is the largest SLC family and includes transporters of dopamine, 5-HT, norepinephrine, glycine and GABA (Gether et al., 2006). SLC6 transporters operate as Na^+ dependent co-transporters that use the transmembrane Na^+ gradient to couple downhill transport of Na^+ with uphill

transport of their substrate from the extracellular to the intracellular environment. It is well established that neurotransmitter transporters have roles in several neurological and psychiatric diseases. Furthermore, the SLC6 family of transporters is a target of several pharmaceutical compounds. For example, GAT-1, which is one member of the SLC6 family, is the target of the antiepileptic drug tiagabine (Schousboe et al., 2004). Interestingly, the member 1 of the SLC6 family, which is responsible to transport GABA, was down regulated by VA (valproic acid) (Table 4B), which has been widely used for treating and preventing certain types of epileptic seizures, and is also used as mood stabilizer in bipolar affective disorders. It is tempting to speculate that this specific induction by VA is linked with the mechanism of antiepileptic capability.

Solute carrier family 16 (SLC16s) which is a transmembrane protein with 12 transmembrane domains is the cotransporter of monocarboxylate, such as lactate, pyruvate and ketone bodies. Lactic acid transport across the plasma membrane is fundamental for the metabolism of lactic acid and pH regulation of all cells, removing lactic acid produced by glycolysis and allowing uptake by those cells utilizing it for gluconeogenesis or as a respiratory fuel. Rapid transport of lactic acid transport across the plasma membrane is of fundamental importance to all mammalian cells under hypoxic conditions when they become glycolytic. To date, no data are available on the properties of member 6 and 9 although member 6 distribution in human tissues has been subject to Northern blot analysis (Halestap et al., 2004).

4.2.3.3 Heat shock proteins

Table 4C Heat shock proteins and chaperones

Gene name	Gene ID	As	CA	Cd	Pb	PCB	tBHQ	VA
Stress protein HSP70	AB062116	10.1	2.2	7.0	2.5	5.4	12.3	2.7
	AF210640	10.9		6.6	2.5	5.0	10.8	2.7
Hsp70 (2)	AF006007	7.6		6.3	2.1	4.6	10.1	2.4
Heat shock protein HSP 90-alpha	AF068773	2.6						
Heat shock cognate 70 kDa protein	BM024785	3.0						
DnaJ (Hsp40) homolog, family A, member 1	BI891737	3.6					3.6	
Ahsa1 protein	BM103957	2.2					2.1	

Heat shock proteins 70 (Hsp70s) assist a wide range of folding processes, including the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of organellar and secretory proteins, and control of the activity of regulatory proteins. Hsp70s have housekeeping function in the cell in which they are built in components of folding and signal transduction pathways, and quality control functions, in which they proof-read the structure of proteins and repair misfolded conformers. All these activities are based on the property of Hsp70 to interact with hydrophobic peptide segments of proteins in an ATP-dependent way. Overexpression of Hsp70 leads to increased resistance against apoptosis-inducing agents such as tumor necrosis factor-α, staurosporin and doxorubicin, while downregulation of Hsp70 levels by antisense technology leads to increased sensitivity towards these agents (Mayer et al., 2004).

In vertebrates, *hsp70s* consist of stress inducible (*hsp70*) and heat shock cognate (*hsc70*). In fact, a PCR based study revealed the *hsp70* gene exhibited strong heat-inducible expression during several stages of zebrafish embryonic development. This gene was also specifically expressed during normal lens development under non stress conditions. Zebrafish *hsc70* is expressed in the developing CNS and a fraction of somites during development and exhibits only a slight increase in expression following heat shock. However, it is strongly induced during fin regeneration (Krone et al., 2003). Vertebrates express three *hsp90* genes, *hsp90a*, *hsp90a2*, and *hsp90b*. The expression of *hsp90a* and *hsp90a2* is restricted primarily to cells in the pre-somitic paraxial mesoderm, somites and pectoral fin buds in zebrafish embryos (Krone et al., 2003; Etard et al., in preparing).

The 96-120 hpf microarray data revealed that *hsp70s* were up regulated strongly by 6 out of 11 chemicals (Table 4C). The induction of *hsp70* expression by exposure to the three heavy metals was coincident with previous studies however *hsp70* has not been regulated significantly by MeHg exposure. The same case was found in DDT and TCDD exposed embryos.

4.2.3.4 Metalloendopeptidase

Table 4D Metalloendopeptidase genes

Gene name	Gene ID	As	4CA	Cd	Hg	Pb	PCB	tBHQ	VA
Matrix metalloproteinase 9	AW174507	2.1		12.3	6.7	3.9	14.2	6.4	
Matrix metalloproteinase 13	AW305943		2.5	11.9	5.9	3.5	15.1	10.2	
Tissue inhibitor of metalloproteinase 3	BM023829						2.5		2.0

The development of a multicellular organism is dependent upon an extracellular matrix (ECM), which facilitates the organization of cells into more complex functional units: tissue and organs. The ECM is the glue that holds cells together, and provides texture, strength and integrity to the tissues. The cleavage of ECM molecules is carried out by specialized proteinases. Among these are the matrix metalloproteinases, a family of zinc- and calcium-dependent endopeptidases that have specificity for a subset of ECM molecules (Thiennu., 2001).

The activities of matrix metalloproteinases are controlled by a combination of proteolytic pro-enzyme activation steps and inhibition by endogenous inhibitors like α2-macroglobulin and the tissue inhibitors of metalloproteinases. (Zhang et al., 2003) Interestingly, only two members, *MMP9* and *MMP13* of zebrafish matrix metalloproteinases were strongly regulated by 7 (As, 4CA, Cd, Hg, Pb, PCB, and tBHQ) out of 11 chemicals (Tab. 4D). On the other hand, the transcription levels of *MMP9* and *MMP13* were not induced significantly by AA, DDT, and TCDD administration.

4.2.3.5 Transcription activity related genes

Suppressors of cytokine signaling (SOCS) form a family of proteins that regulate the strength and duration of the cytokine signaling cascade. Eight SOCS family members have been identified thus far, and these include CIS and SOCS1-7. Molecules in this family contain a SH2 domain and a segment called SOCS box located near the C terminal end. Both of these domains are thought to be required for proper function, which is to

bind to the cytokine receptor or associated JAKs (Janus-kinases) and to attenuate signal transduction directly and by targeting the receptor complex for ubiquitin-mediated degradation in proteasomes. SOCS3 has been regarded as a negative regulator of inflammation and inhibitor of the angiotensin-activated JAKs-STAT (signal transducer and activator of transcription) pathway. SOCS3 is not only regulated by cytokines, including inflammatory cytokines such as tumor necrosis factor and IL-1, but also by Toll like receptor ligands such as lipopolysaccharide. There is increasing evidence that SOCS3 is critical in the pathogenesis of autoimmune disease (Gan et al., 2005). The expression of SOCS3 was up regulated strongly by 5 (4CA, Cd, MeHg, PCB, tBHQ) out of 11 chemicals (Table 4E). This suggests that these chemicals induce cytokine signaling in some form.

Table 4E Transcription activity related genes

Gene name	Gene ID	As	CA	Cd	Hg	Pb	PCB	tBHQ	VA
Activating transcription factor 3	AW422298	4.0		3.2	3.3	2.5	15.1	5.1	5.4
Zinc fingers and homeoboxes 1	AI793802	2.5		2.6	2.7	2.4	4.2	2.1	2.0
CCAAT/enhancer binding protein (C/EBP) beta	AW019436	2.1		2.3		2.4	6.9	2.3	
Suppressor of cytokine signaling 3	BG727181		2.0	2.5	4.5		5.6	3.7	
	BI878700			2.2	2.7		3.4	3.1	
Interferon regulatory factor 1	BI326597		2.1	2.7	2.1				2.3
Interferon regulatory factor 7	BE605965		2.3				7.9		6.8

The interferon-regulatory factor (IRF) family of transcription factors was initially found to be involved in the induction of genes that encode type I interferons. IRFs have been shown to have functionally diverse roles in the regulation of the immune system with their interaction with toll-like receptors and other pattern-recognition receptors. The mammalian interferon-regulatory factor (IRF) family comprises nine members: IRF1-IRF9. Each IRF contains a well conserved DNA binding domain of ~120 amino acids; this is located at the amino terminus and forms a helix-turn-helix motif. This region recognizes a consensus DNA sequence that is known as interferon (IFN)-stimulated response element. The carboxy-terminal regions of IRFs, except IRF1 and IRF2, show homology to the C-terminal domains, which mediates the response to the cytokine transforming growth factor-β. IRF1 has the function to activate promoters in type I interferon genes. IRF7 is expressed in small amounts in most cells and is strongly

induced by type I IFN mediated signaling. IRFs have been shown to have functionally diverse roles in the regulation of the immune system. The crucial involvement of IRFs in innate and adaptive immune responses has been attention with the discovery of their role in immunoregulation by Toll-like receptors (Honda et al., 2006). The expression of interferon regulatory factor 1 and 7 were induced strongly by 4CA, Cd, MeHg, PCB, and VA. These gene expression profiles suggest that the immune system has been induced by these toxicants.

The CCAAT/enhancer binding proteins (C/EBP) are a family which consists of six members sharing numerous structural and functional features. The prototypic C/EBP is a modular protein, containing a carboxy-terminal leucine-zipper dimerization domain, a DNA binding domain and an *N*-terminal activation domain. Dimerization via the leucine zipper region, with homo- or heterotypic C/EBPs or other transcription factors is a prerequisite for DNA binding and subsequent gene activation. All the members, with the exception of C/EBPε, are expressed in multiple cell types but protein levels vary with developmental stage and tissue examined (Lekstrom-Himes., 2001). C/EBPβ has both positive and negative functions in regulation of cell division and transcription by interaction with other transcription factors. The expression level of this gene was up regulated significantly by As, Cd, Pb, PCB, and tBHQ exposures (Table 4E). Therefore, overexpression C/EBPβ should induce a wide range of changes in gene expression.

4.2.3.6 Monooxygenase

Cytochrome P450s (Cyp 450s) form a large and diverse superfamily of genes that code for enzymes involved in the oxidative metabolism of various xenobiotics and endogenous molecules. The expression of many Cyp450 genes is modulated by xenobiotics, and the regulation can take place at different levels ranging from transcriptional activation to protein stabilization. The transcriptional activation of Cyp450 genes has been widely studied. A number of xenobiotic activated receptors that act as specific transcription factors, have been discovered. The components of the aryl hydrocarbon receptor2 (AHR2) and aryl hydrocarbon receptor nuclear translocator1 (ARNT1) signaling pathway have

been identified and functionally characterized for mediating TCDD developmental toxicity in zebrafish embryos (Carney et al., 2006). The expression of cytochrome p4501A1 and p4501B is mediated through AHR2/ARNT1 signaling pathway via induction by the aromatic hydrocarbons like TCDD. In agreement, the microarray results revealed that mRNA level of *Cyp4501A1* was up regulated by exposure to 4CA, DDT, PCB, tBHQ, and TCDD. Moreover, the expression of *Cyp4501B* and aryl hydrocarbon receptor2 was also up-regulated by TCDD.

4.3 Confirmation of microarray data

The number of genes regulated significantly by the 11 toxicants exceeds 540 for the exposure regimen between 96 to 120 hpf. The changes in expression of these genes were scored as statistically significant different with $P_{adj}<0.025$, $|FC|>2$ (See 3 chapter). To obtain a rough idea of the number of false positives, I used the semi quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to confirm these expression changes for selected genes that were induced by different toxins in the microarray studies. As a second method, whole mount in situ hybridization was applied to confirm the microarray data and to study the gene expression patterns.

4.3.1 RT-PCR (Reverse transcriptase polymerase chain reactions)

The microarray data were confirmed by previously observed induction of *cyp 1a1* and *hsp 70* in zebrafish embryos exposed to TCDD and Cd respectively (Tanguay et al; Krone et al., 2003). In order to evaluate the proportion of significantly regulated genes, *cyp 1a1* and *hsp 70* were used as reference genes while the β-actin served as an amplification control. For the remaining 12 genes that were checked by RT-PCR, no expression data from exposure studies were available. Using semi quantitative RT-PCR the differential expressions of these genes compared with vehicle controls were analyzed in replicated experiments (Figure 4.6). The keratin type I gene (AI397347) transcription was down regulated 2.6 times, and, *cyp 1a1* (AF057713) was up regulated 37.7 fold in microarray experiment with cDNA from TCDD treated larvae. These changes in gene

expression were confirmed by RT-PCR indicating a clear correlation with the microarray data (Figure 4.6).

The microarray data indicated that the expression of *parvalbumin3a* (BE201681), *matrix metalloproteinase 9* (AW174507) and *13* (AW305943), *thioredoxin* (BI864190) and *Hsp70* were altered in zebrafish embryos exposed to Cd. The expression of the four genes, *parvalbumin3a, matrix metalloproteinase 9* and *13, thioredoxin* were clearly found to be regulated by Cd exposure also with the RT-PCR method. The inductions of 12 genes in embryos exposed to 7 toxicants between 96 to 120 hpf were confirmed and showed a correlation with the microarray data (Figure 4.6). The gene (BM183152) which is the zebrafish heterogeneous nuclear ribonucleoprotein A/B and was up regulated by TCDD exposure has not been amplified successfully by RT-PCR.

Toxicants	Gene ID	Gene name	Fold change	cycle numbers
TCDD	AI397347	Similarity to keratin type 1 (human)	-2.6	30
	AF057713	Danio rerio cytochrome p 4501A	37.7	25
DDT	BI533854	Weakly similar to c-type lectin	-2.1	25
Cd	BE201681	Danio rerio Oncomodulin A	-4.4	25
	AW174507	Danio rerio materix metalloprotinase 9	8.8	25
	AF210640	Danio rerio HSP 70	8.2	20
	AW305943	Danio rerio materix metalloproteinase 13	7.5	30
	BI864190	Similarity to thioredoxin	4.3	25
Hg	AW232474	Danio rerio glutathione peroxidase 1	-4.7	25
	BI980610	Similarity to natural killer cell enhacning factor	3.9	25
	BI864190	Similarity to thioredoxin	3.8	25
	BG727181	unknown	3.2	30
VA	AY050500	Danio rerio cone transducin alpha subunit	-2.4	25
	AW422298	Similarity to transcription factor ATF-3	4.2	25
4 CA	BI843145	unknown	-2.8	30
	BI980610	Similarity to natural killer cell enhancin g factor	3.9	30
As	BI864190	Similarity to thioredoxin	11.4	30
Embryo medium	β-actin		ND	30
0.2% ethanol	β-actin		ND	30
0.025%DMSO+1.4 mg/l toluene	β-actin		ND	30

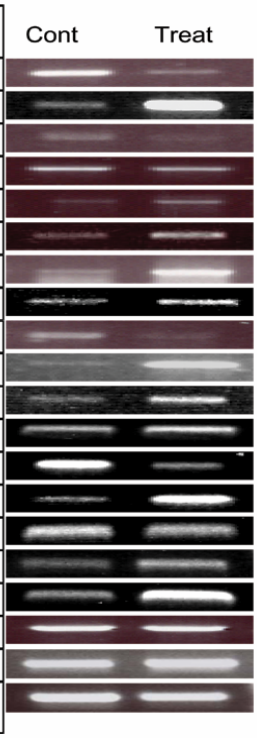


Figure 4.6 RT-PCR analysis confirms selected gene responses. Embryos were exposed with the indicated toxins (500 ng/l TCDD; 15 mg/l DTT; 5 mg/l Cd; 60 µg/l MeHg; 50 mg/l VA; 50 mg/l 4CA; 79 mg/l As) between 96 and 120 hpf. cDNA was synthesized and subjected to PCR with gene specific primers. The number of temperature cycles (cycle numbers) for every set of amplification is indicated. The column "Fold change" summarizes the results from the microarray experiments for comparison with the RT-

PCR results shown in the right panel. β-actin mRNA was used as a toxin insensitive reference. Cont, vehicle-treated embryos, Treat, toxin treated embryos.

4.3.2 In situ hybridization

Selected genes, like *cytochrome p450 1A1 (cyp1a1)* (AF057713), *parvalbumin 3a* (BE201681), *matrix metalloproteinase 9 (mmp9)* (AW174507), *matrix metalloproteinase 13 (mmp13)* (AW305943), *thioredoxin* (BI864190) and *glutathione peroxidase 1* (AW232474) were amplified by RT-PCR and subcloned into plasmid (pGEMT easy, pCS2+). After confirmation by sequencing, the antisense RNA probes were labeled with digoxigenin or fluorescein and used for in situ hybridization on whole mounts.

Low levels of *cyp 1a1* mRNA are expressed normally in the heart and liver (Tanguay et al., 2003). *cyp 1a1* mRNA levels were strongly elevated in the vasculature, heart and liver after exposure of embryos to TCDD for one hour just after fertilization (Tanguay et al., 2003). A similar induction was observed in embryos treated from 4 to 48 hpf with 150 ng/l TCDD. Interestingly, *cyp 1a1* mRNA is highly induced in the vasculature but it was not triggered in heart and liver (Figure 4.7 A, B). *Glutathione peroxidase 1* mRNA is expressed in the gut (Figure 4.7 C) but its expression was completely abolished in embryos exposed to MeHg (Figure 4.7 D), in agreement with the microarray data and the RT-PCR analysis.

The expression of *parvalbumin 3a* mRNA in 24 hpf occurs in mucous cells, the olfactory placode, otic vesicle and anterior pituitary. Its expression can be found in the anterior lateral line neuromast at 36 hpf. At 72 hpf, all mature neuromasts in both the anterior and posterior lateral line systems robustly express *parvalbumin 3a*. In anterior neuromast, *parvalbumin 3a* is expressed in both sensory hair cells and supporting cells (Hsiao et al., 2002). Interestingly, *parvalbumin 3a* expression is decreased dramatically in the neuromasts of the anterior and posterior lateral line (19 embryos with decreased expression out of 20 embryos examined) after exposure of embryos to Cd (500 µg/l) from 4 to 72 hpf (Figure 4.7 E, F), in agreement with the microarray data and the PCR results.

Additional expression domains of *thioredoxin* were noted after treatment of embryos with Cd (500 µg/l) from 4 to 72 hpf and from 96 to 120 hpf. The exposed embryos reveal high levels of *thioredoxin* transcripts in the neuromasts of the anterior and posterior lateral line and olfactory epithelium (Figure 4.7 G, H). Higher magnification indicates that *thioredoxin* mRNA is specifically expressed in the hair cells while no expression was detected in support and mantel cells (12 embryos with increased expression out of 13 embryos)

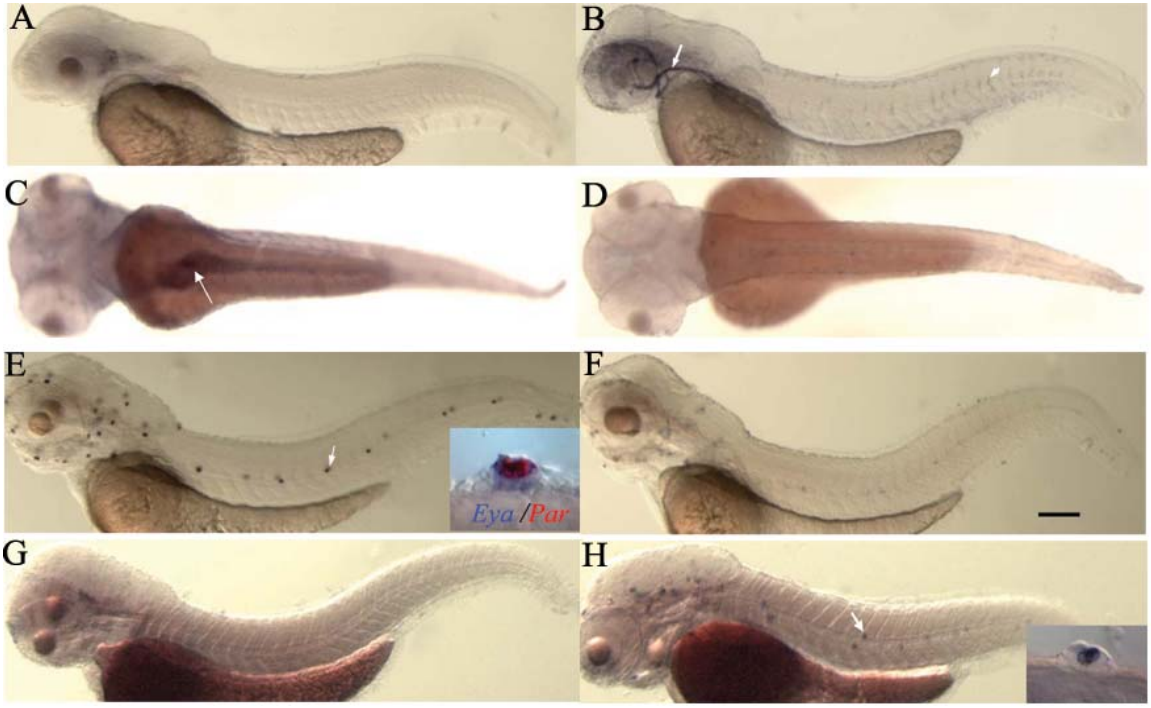


Figure 4.7 In situ hybridization A, B: Vehicle control (A) and 150 ng/l TCDD-treated embryos (B) (48 hpf) hybridized to a *cytochrome P450 1A1* antisense probe. TCDD treated embryos showed increased levels of *cytochrome P4501A1* mRNA in blood vessels (24/25 embryos) (arrow, primary head sinus, arrow head, intersegmental vessel). C, D: 72 hpf control and 60 µg/l MeHg exposed embryos hybridized to *the glutathione peroxidase 1* probe. Embryos showed a reduction of mRNA levels in the gut (14/15) (arrow). E, F: Control embryo (E) and 500 µg/l $CdCl_2$-treated embryo (F) hybridized to a *parvalbumin 3a* antisense mRNA. *parvalbumin 3a* mRNA levels are down-regulated in the hair cells and support cells of the lateral line organ in response to Cd exposure (19/20 embryos). At the right corner is a high magnification of one neuromast. Eya mRNA is expressed in both hair cells and support cells (blue). *parvalbumin 3a* mRNA is expressed also in both hair cells and support cells (red). G, H: Control embryo and 500 µg/l $CdCl_2$-treated embryos hybridized to a *Thioredoxin* antisense probe. Embryos showed an increased expression of mRNA levels in the lateral line (12/13 embryos) (arrow). The right corner is the high magnification of one neuromast. *Thioredoxin* mRNA expression

can be detected only in the hair cells. Embryos were treated from 4 hpf to 72 hpf and were then fixed for in situ processing. Embryos are oriented anterior to the left and dorsal up (A to H) or view onto dorsal (C, D). Scale bar represents 220µm.

4.4 Gene expression profiles of different exposure doses

The assessment of hazardous chemicals is currently predominantly based on the morphological endpoints or lethality in 50% of cases. In fact, many severely adverse effects may be induced by toxicants at concentrations lower than the EC50. In particular, low dose chronic exposure over longer time periods may cause serious pathological effects.

The doses of the toxicants to which embryos were exposed in the initial experiments, were adjusted so that the chemicals caused a morphological toxic effect among approximately 50 percent of total treated embryos. Exposure to the lower doses (see appendix A) did not cause morphologically visible defects in most cases. I wanted to know whether I could still measure changes in the expression profiles in response to these low doses. I exposed embryos from 96 to 120 hpf to either 50 percent (4CA, Cd, TCDD, VA, MeHg) or 10 percent (4CA, DDT, Cd, TCDD, VA, MeHg) or 1 percent (4CA, DDT, Cd, VA) of the original concentration.

4.4.1 Comparing gene expression profiles at lower doses

The gene expression profiles at different cadmium concentrations revealed that the number of significant gene responses decreased with the reduced concentrations. For instance, the number of induced genes (P_{adj} <0.025 and |FC|>2.0) were decreased from 121, 49 to 30 at 5 mg/l, 2.5 mg/l, and 0.5 mg/l Cd, respectively. Although, the fold-induction of specific genes tended to be reduced with decreasing cadmium concentrations, many genes retained an induction at 10× lower concentration. For example, the expression of *GTP cyclohydrolase 1* (AJ311846) was down-regulated 3.3-, 2.7-, and 2.6-fold by administration of 5 mg/l, 2.5 mg/l, and 0.5 mg/l Cd, respectively. The mRNA level of *thioredoxin* (BI864190) was up-regulated 4-, 2.9-, and 2.2-fold by 5 mg/l, 2.5

mg/l, and 0.5 mg/l Cd, respectively. Furthermore, the expression of *arginase II* (BF717769) and *heat shock cognate 70* (AF210640) were regulated by exposure to 5 mg/l and 2.5 mg/l Cd. However, they were not induced significantly with 0.5 mg/l Cd (Table 5A). Similarly, an induction of *MMP9* and *MMP13* transcription was only found when embryos were exposed to 5 mg/l Cd. It is clear that the number of induced genes but also the foldness of induction was higher at high concentrations. Moreover, these data suggest that thresholds exist for the induction of particular genes.

Table 5A Comparing genes expression at different doses

Name	ID	Cd (5 mg/l)	Cd (2.5 mg/l)	Cd (0.5 mg/l)
GTP cyclohydrolase 1	AJ311846	-3.3	-2.7	-2.6
Thioredoxin	BI864190	4.0	2.9	2.2
unknown	AW019526	-2.5	-2.0	-2.1
unknown	BI887138	2.5	2.5	2.5
Ribosomal protein S17	BI474700	8.6	8.5	4.8
Parvalbumin3a /oncomodulin A	BE201681	-4.3	-5.7	-4.5
Solute carrier family 16, member 9	BE016639	2.9	2.4	2.5
Peroxiredoxin 1	BI980610	3.1	2.8	2.3
arginase, type II	BG891983	3.1	2.3	2.1
Alpha-2-HS-glycoprotein	AI496863	-3.3	-2.7	-2.9
sequestosome 1	AW343560	3.9	3.1	2.5
Arginase, type II	BF717769	2.9	2.1	
heat shock cognate 70-kd protein	AF210640	6.6	2.9	
unknown	AI353541	2.8	2.5	
unknown	BG727177	2.2	2.1	
Sulfide quinone reductase-like	BI882244	3.6	3.0	
uncoupling protein 2	AJ243250	2.3	2.5	
Tripartite motif-containing 62	BI878269	2.2	2.4	
Collagen, type VII, alpha 1	AI601501	-2.3	-2.3	
Total Regulated genes number		121	49	30

The microarray results of MeHg exposures at different doses showed explicitly that the number of induced genes were 144, 14, and 5 in embryos treated with 60 µg/l, 30 µg/l, and 6 µg/l MeHg respectively. Moreover, the mRNA levels induced by MeHg exposures were correlated with the concentration of MeHg. For example, the expression level of *peroxiredoxin* (BI980610) was up-regulated 7.7-, 4.6-, and 3.6-times by 60 µg/l, 30 µg/l, and 6 µg/l MeHg significantly. Furthermore, the mRNA of *MMP13* (AW305943) was triggered by MeHg exposures at 60 µg/l and 30 µg/l doses but it was not induced by 6 µg/l MeHg. Similarly, the expression of *parvalbumin3a* (BE201681) was repressed by 60

µg/l and 30 µg/l but not by 6 µg/l MeHg. The same pattern of expression was found for *glutathione peroxidase2* (AW232474) and *glutathione peroxidase4a* (BI896246) genes in response to the different MeHg concentrations (Table 5B). Additional 131 response genes were scored in embryos exposed to 60 µg/l MeHg. Examples are *MMP9* (AW174507), *ATF3* (AW422298), and *C/EBPα*. The gene expression profiles demonstrated that specific gene responses can be identified successfully at low concentrations of toxicants that do not cause significant morphological effects.

Table 5B Comparing genes expression at different doses

Name or Human Homology Gene	ID	Hg (60 µg/l)	Hg (30 µg/l)	Hg (6 µg/l)
Ribosomal protein S17	BI474700	6.9	4.2	2.2
Peroxiredoxin	BI980610	7.7	4.6	3.5
Glutamate-cysteine ligase	BG304082	2.4	2.1	
Matrix metalloproteinase 13	AW305943	5.9	2.8	
ATP-binding cassette, sub-family C. 2	AW279805	2.4	2.2	
Parvalbumin 3a /oncomodulin A	BE201681	-2.6	-2.3	
Glutathione S-transferase omega 1	AW019036	2.6	2.7	
Apical early endosomal glycoprotein precursor	BG302634	3.0	2.4	
Glutathione peroxidase 2	AW232474	-4.2	-3.0	
Glutathione peroxidase 4a	BI896246	-2.6	-2.8	
Glutathione S-transferase pi	AF285098	3.2	2.2	
Suppressor of cytokine signaling 3	BG727181	4.5	2.4	
unknown	AI397362		2.8	2.7
The total number of regulated genes		144	14	5

TCDD is a persistent, lipophilic environmental contaminant that bioaccumulates in fish, which is the most potent and the prototype inducer for the study of the common aryl hydrocarbon receptor mediated gene regulation. In fact, the microarray results of TCDD exposed embryos at different concentrations revealed that *cytochrome p450 1a1* (AF057713) is a very sensitive marker gene that can be induced significantly by 50 ng/l TCDD. Furthermore, the expression of *cytochrome P4501B* (BG738243) was up-regulated by TCDD at 500 ng/l and 250 ng/l doses but no significant induction was found at 50 ng/l TCDD (Table 5C). Zebrafish *aryl hydrocarbon receptor2* (AF063446) was up-regulated 1.9 and 1.8 times by exposures to 500 ng/l and 250 ng/l TCDD, respectively but no significant induction could be demonstrated at 50 ng/l. In this case, the parameters to choose significant response genes were $|FC|>1.5$ $P_{adj}<0.025$. The reason is that the fold-

changes of transcription factors were not so large that they could be picked up under a higher exclusion limit of fold changes. The majority of genes with a high fold-change should be representative gene targets of the signaling pathways. For example, *cyp450 1a1* and *cyp450 1b* are regulated directly by the AHR2/ARNT1 signaling pathway.

Table 5C Comparison of gene expression at varying concentrations

Name or Human HomoloGene	ID	TCDD (500 ng/l)	TCDD (250 ng/l)	TCDD (50 ng/l)
Cytochrome P450 1A1	AF057713	31.6	29.5	5.0
Keratin 15	AI397347	-2.2	-2.4	
Similar to SULT6B1	AI959735	2.8	2.3	
cytochrome P450 1B	BG738243	12.9	11.3	
unknown	BM183152	3.3	3.0	
unknown	BM101698	2.2	2.3	
unknown	AI397362		2.8	2.7
unknown	AI384221	2.1		
Forkhead box Q1	AW566603	2.1		
Gastric intrinsic factor	AI353694	2.1		
Cytochrome c oxidase subunit IV isoform 2	BI672330	2.3		
unknown	AI353244		-2.1	
Arrestin domain containing 3	BF157296		-2.1	
unknown	BI326783		-2.2	
unknown	AI958860		-2.1	
Apical early endosomal glycoprotein precursor	BG302634		3.3	
Arginase, type II	BG891983		-2.2	
unknown	AW019312			2.1
The total numbers of regulated genes		10	13	3

The principal component analysis shows explicit clusters of different toxicants but shows similar Eigenvalues between different exposure doses of one chemical. For example, the scatter plots of TCDD and VA are clustered far from the position of the other toxicogenomic patterns but the toxicogenomic patterns induced by the three different doses are close to each other (Figure 4.8) indicating similarity of gene responses.

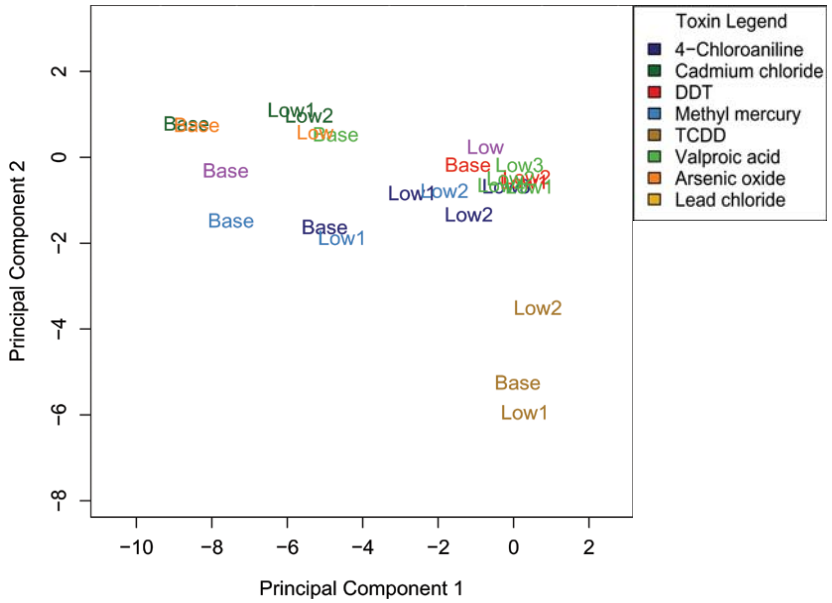


Figure 4.8 Principal component analysis of low doses exposure. Principal component analysis showing gene responses in embryos treated between 96 and 120 hpf with 4CA (Base, 50 mg/l, Low1, 25 mg/l, Low2, 5 mg/l, Low3, 0.5 mg/l); Cd (Base, 5 mg/l, Low1, 2.5 mg/l, Low2, 0.5 mg/l); DDT (Base, 15 mg/l, Low1, 1.5 mg/l, Low2, 0.15 mg/l); MeHg (Base, 60 µg/l, Low1, 30 µg/l, Low2, 6 µg/l); TCDD (Base, 500 ng/l, Low1, 250 ng/l, Low2, 50 ng/l); VA (Base, 50 mg/l, Low1, 25 mg/l, Low2, 5 mg/l, Low3, 0.5 mg/l); As (Base, 79 mg/l, Low, 7.9 mg/l) and Pb (Base, 2.8 mg/l, Low 0.28 mg/l). The 8 clusters are separated from each other but the gene expression profiles induced by the same chemical at different concentrations are similar as expected.

Hierarchical clustering of gene response (Figure 4.9) in embryos treated between 96 to 120 hpf with TCDD in different doses shows similar patterns of gene inductions but the foldness of induction of the genes decreased at lower doses. For example, *cytochrome p450 1A1* (AF057713) was induced 32, 30 and 5-times by 500 ng/l, 250 ng/l and 50 ng/l TCDD, respectively, compared with vehicle treatments. The mRNA level of *Cyp4501B* (BG738243) was up-regulated 13 and 11 times in 500 ng/l and 250 ng/l TCDD treated embryos while *Cyp4501B* mRNA was not significantly increased by 50 ng/l TCDD. While Cd, TCDD, MeHg and Pb show significant gene responses at the lowest concentrations tested, the other compounds did not trigger very strong responses at the lowest concentrations suggesting that these are below the no-effect-concentrations

(NOEC). To establish the NOEC however precisely, I would need to carry out a much more fine grained dose response analysis which was unfortunately not possible due to the enormous cost involved to carry out such an analysis.

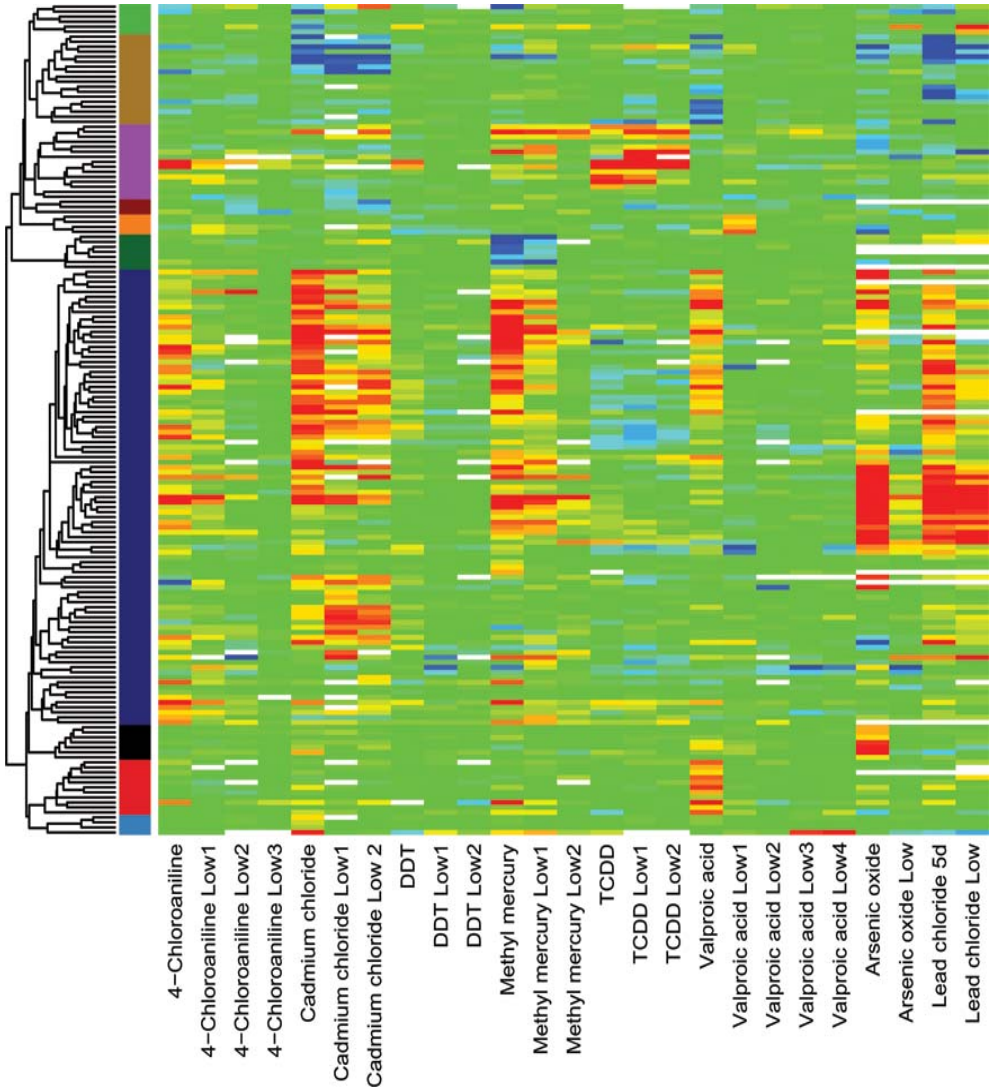


Figure 4.9 Gene expression profiles of low doses. Expression profiles of induction in embryos treated between 96 and 120 hpf with the 4CA (50 mg/l, Low1, 25 mg/l, Low2, 5 mg/l, Low3, 0.5 mg/l); Cd (5 mg/l, Low1, 2.5 mg/l, Low2, 0.5 mg/l); DDT (15 mg/l, Low1, 1.5 mg/l, Low2, 0.15 mg/l); MeHg (60 µg/l, Low1, 30 µg/l, Low2, 6 µg/l); TCDD (500 ng/l, Low1, 250 ng/l, Low2, 50 ng/l); VA (50 mg/l, Low1, 25 mg/l, Low2, 5 mg/l, Low3, 0.5 mg/l); As (79 mg/l, Low, 7.9 mg/l); and Pb (2.8 mg/l, Low 0.28 mg/l). The 8 clusters indicated explicitly but sub-clusters that induced by one chemical in different doses showed similar correlation with each other.

4.5 Gene expression profiles of mixture

In the environment, organisms are rarely exposed to individual chemicals, and assessment of toxic effect has therefore to take into account the co-occurrence of diverse contaminants with different toxic potentials. Toxicologists are thus faced with the enormous task to assess complex chemical mixtures that may have synergistic toxicological effects on the ecosystem.

Chemicals may behave different in mixtures leading to independent, additive, synergistic or antagonistic toxic effects. If two or more chemicals have different target sites in different processes, their effect can be expected to be independent. Mixtures of chemicals with a common targets site and the same mode of action act according to concentration additively. If the components of the mixture interact with each other, they might cause antagonistic or synergistic effects (Escher et al., 2002). A series of studies, where the full concentration effect curves of a large number of single compounds and mixtures of compounds, with similar and dissimilar mode of action, are performed previously. The effect of mixtures, however, has not been assessed in gene expression profiles.

Next, it was tested whether synergistic effects of compound mixtures can be observed in gene expression profiles. To provide proof-of- principle, I exposed embryo between 96 to 120 hpf to a mixture of low concentrations of Cd (50 µg/l), Pb (280 µg/l), MeHg (6 µg/l), and As (7.9 mg/l). About twofold more genes (167) were significantly regulated by administration of the mixture in comparison to the sum of the genes significantly regulated in response to exposure with the individual toxins (Figure 4.10). Moreover, in most cases the induced mRNAs levels were higher in response to the mixture than the changes in gene expression levels triggered by individual chemicals. For instance, the mRNA levels of *heat shock protein 70 genes* (AB062116, AF210640, AF006007) were dramatically up regulated by the mixture compared with the control but they were not significantly regulated by individual toxins. In the case of *peroxiredoxin* (BI980610), the mixture induced a strong increase by 16 times in transcript level, while mRNA levels were up-regulated 3-, 3.5- and 10-times by As, MeHg and Pb, respectively, while *peroxiredoxin* mRNA was not significantly regulated by Cd. *Parvalbumin3a* (BE201681)

mRNA was down regulated 4.3-, 3.5- and 5.5-fold by Cd, Pb and the mixture, respectively, but it was not significantly affected by As and MeHg. Antioxidant defence systems, like the glutathione and thioredoxin systems were up-regulated strongly by the mixture (Table 6).

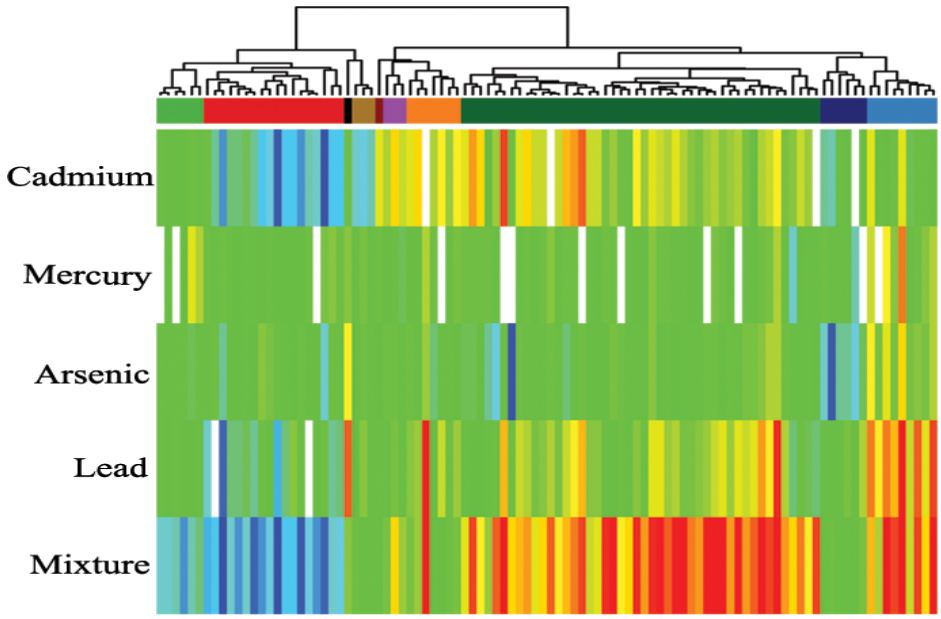


Figure 4.10 Gene expression profiles of mixture. Embryos were exposed either to Cd (50 µg/l) or MeHg (6 µg/l) or As (7.9 mg/l) or Pb (280 µg/l) alone or to a mixture of Cd, (50 µg/l); MeHg (6 µg/l); As (7.9 mg/l); Pb (280 µg/l). The mixture shows a strongly increased response with respect to the degree of changes of individual genes. The blank indicates the missing data.

In summary, the number of genes regulated by the mixture was more than additively increased. Interestingly, the expression of some genes while affected by individual chemicals was not significantly altered by the mixture suggesting antagonistic effects. For example, *period homolog2* (BG303941) was up-regulated by As, Cd, and Pb but it was not induced significantly by exposure to the mixture. However, this needs to be verified by an independent method.

Table 6 Comparing selected genes expressions at mixture

Name or Human homology Gene	ID	As	Cd	Hg	Pb	Mixture
Peroxiredoxin	BI980610	2.7		3.5	9.6	15.5
Gastric intrinsic factor	AI353694	2.0		2.4	3.3	5.1
kruppel-like factor 2a (klf2a)	AF392992	-2.1	-4.8			-3.9
Period homolog 2 (Drosophila)	BG303941	2.4	2.1		3.5	
Ribosomal protein S17	BI474700		4.3	2.2		10.5
Parvalbumin 3a / Oncomodulin A	BE201681		-4.3		-3.0	-5.5
uncoupling protein 2 (ucp2 gene homologue).	AJ243250		2.1		2.2	4.5
CCAAT/enhancer binding protein (C/EBP), beta	AW019436	-2.3				4.0
Suppressor of cytokine signaling 3	BI878700	-4.9				2.6
Matrix metalloproteinase 13 (collagenase 3)	AW305943		3.2			4.1
Arrestin domain containing 3	BF157296		2.5			2.7
unknown	BI888424		2.1			2.1
Complement component 7	AA497156		4.1			10.1
Stathmin 1/oncoprotein 18	BI891936		-2.2			-3.2
Solute carrier family 16 member 9	BE016639		3.3			3.5
Solute carrier family 16 member 6	AW421040		4.0			4.3
unknown	AW019503		2.2			4.3
gelsolin	AF175294		-2.1			-2.8
DnaJ (Hsp40) homolog, subfamily A, member 1	BI891737		2.3			4.7
unknown	BQ618331		2.5			3.2
unknown	BI325662		-2.1			-3.1
unknown	AL591382		2.5			2.3
Keratin 25D	AI397347		-2.2			-2.4
Solute carrier family 16 member 9	BI474827		2.7			2.8
unknown	BI886268		-2.3			-2.6
Parvalbumin	BI845755		-3.0			-4.1
ornithine decarboxylase	AF290981		2.1			2.9
Activating transcription factor 3	AW422298		2.1			10.2
Alpha-2-HS-glycoprotein	AI496863		-3.2			-6.7
Suppressor of cytokine signaling 3	BG727181		2.2			2.2
thymidylate synthase	AY005804		-2.3			-2.7
Thioredoxin	BI864190				4.7	14.4
unknown	BM182280				2.1	5.5
Glutathione S-transferase omega 2	BI979918				3.7	4.2
unknown	AW115782				2.8	4.5
glutathione S-transferase pi	AF285098				4.3	6.0
Complement component 3	AI588354				2.1	4.0

4.6 Blind test study

Taken together my results suggest that individual chemicals induce characteristic patterns of gene expression. As an independent verification, I carried out blind tests to identify the chemicals by their induced gene expression profile. This was also intended to verify and

to assess the reproducibility of the patterns of expression that were deduced in the previous experiments. The blind test paradigm, the wet part of the microarray analysis was done by one person and the gene expression profile was calculated and compared by a second person without knowledge of the chemicals used. Fourteen out of 15 chemicals were unambiguously identified. In the case of 4CA, close matches were scored to the 4CA, the DDT and the AA response profiles (Figure 4.12). Thus it was in the same group (Figure 4.11).

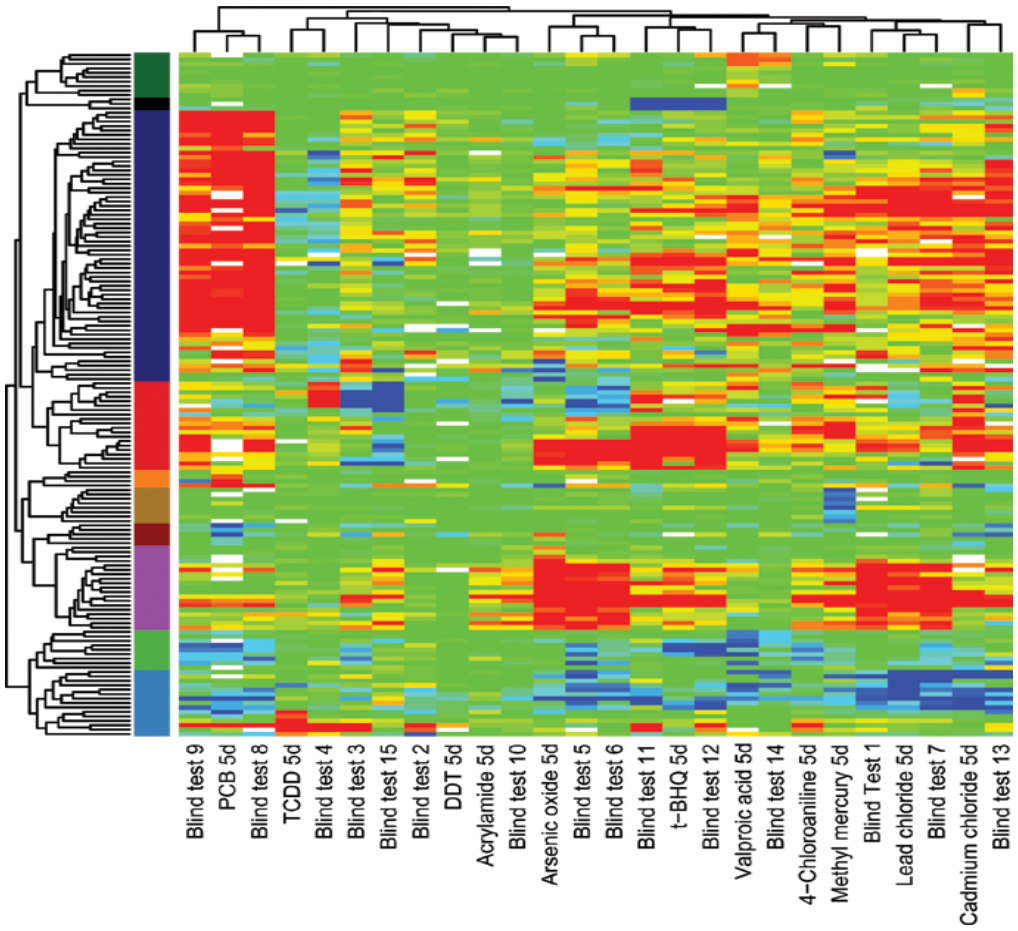


Figure 4.11 Gene expression profiles of blind tests. Embryos were treated between 96 and 120 hpf with 4CA (50 mg/l); Cd (5 mg/l); DDT (15 mg/l); MeHg (60 µg/l); TCDD (500 ng/l); VA (50 mg/l); As (79 mg/l); Pb (2.8 mg/l); PCB (33 mg/l); AA (71 mg/l); tBHQ (1.7 mg/l). All the blind tests were done with the same concentration as in the original experiments.

Test	4CA	Cd	DDT	Hg	TCDD	VA	AA	As	Pb	PCB	tBHQ
Pb	6.15	6.78	8.66	6.76	10.91	7.96	7.62	6.32	5.01	10.66	6.81
DDT	6.74	9.86	4.51	8.79	7.79	8.01	5.39	10.38	8.96	10.05	9.13
4CA	6.87	10.96	6.69	9.47	8.82	8.87	6.99	11.21	8.42	10.75	9.42
TCDD	8.55	12.43	6.78	11.01	5.42	10.37	7.61	10.76	12.61	15.63	10.62
As	9.14	9.56	10.93	9.74	12.46	9.89	10.18	6.88	7.51	11.32	7.51
Pb	7.25	7.95	10.05	7.38	12.06	8.62	8.65	9.58	4.22	9.91	7.70
PCB	9.84	9.30	13.17	10.23	15.01	10.01	11.78	12.77	10.62	3.93	10.40
As	9.18	9.99	9.92	10.20	11.53	10.16	9.43	5.48	7.84	12.40	7.67
tBHQ	8.79	7.49	11.32	9.24	11.52	10.29	10.98	8.93	9.77	11.31	6.33
PCB	8.37	7.74	12.06	8.88	13.65	8.54	10.63	11.79	10.22	5.71	9.08
AA	7.18	10.79	4.29	9.28	7.21	8.63	3.56	7.67	8.12	13.04	8.86
tBHQ	7.49	7.26	11.22	8.30	11.66	9.00	10.40	9.57	9.58	10.47	5.41
Cd	8.32	6.67	10.76	8.39	12.79	8.90	10.16	10.89	6.96	8.05	8.15
VA	6.43	8.25	6.47	7.57	9.04	4.57	6.15	9.56	8.02	10.24	8.33
AA	10.26	14.38	7.09	12.23	9.61	11.59	6.25	11.26	10.54	13.89	11.84

Figure 4.12 Euclidean distance of blind tests Embryos were exposed from 96 to 120 hpf with the test compounds (Test). The table gives the Euclidian distances between the expression profiles of the test compounds and the 11 toxins. The yellow label indicates the correct match. An ambiguous score was obtained in the case of 4CA (grey boxes) with three closely matching expression profiles.

4.7 Molecular mechanism of toxicity

Chemicals can induce proximal and distal gene expression changes in the exposed organism, only some of which play an important role in the toxic mechanism. Other gene responses may be adaptive, protecting the organism from the harmful impact of the chemicals. The functional characterization of these changes in signatures of gene expression will allow a greater understanding of the mechanism by which individual toxicants lead to physiological perturbations. According to the microarray data, *matrix metalloproteinase (MMP) 9* and *13* are highly induced in embryos treated with cadmium and methylmercury. I thus embarked on a more detailed investigation of *MMP 9* and *13* to assess their role in the observed teratological effects of Cd and MeHg treatment.

4.7.1 Methylmercury induces morphological changes at very low concentrations in the zebrafish embryo

Defects and morphological changes were observed in embryos that were exposed to methylmercury either from 4 to 48 hpf or 4 to 72 hpf or 4 to 96 hpf. Abnormal fin shapes and pigmentation were detected, especially the tail fin revealed a difference compared with a control embryo (Figure 4.13 A, C). 43 out of 50 exposed embryos (50 µg/l) showed abnormal fin shapes at 72 hpf. The pigmentation in the ventral tail near the end of the notochord forms a gap in control embryos. This gap in pigmentation was not evident in embryos treated with methylmercury (31/50 embryos) (Figure 4.13 A, C).

In a parallel study, it was shown that this gap was also closed in zebrafish mutants or knock-down embryos, in which the Shh signaling pathway is impaired. Interestingly, a GFP transgene is specifically expressed in this gap of pigmentation. Expression of the transgene is also dependent on a functional Shh signalling pathway. Hadziev et al (submitted) suggested that this region is implicated as growth zone from which the tail fin grows out during late embryogenesis and larval stages. I investigated next whether the expression of the transgene would also be affected by administration of MeHg. Transgenic embryos (*–2.4shh:gfp ABC line 15*) were exposed to MeHg 50 µg/l from 4 to 48 hpf and GFP fluorescence was monitored in live embryos at 96 hpf. MeHg exposure abolished the expression of the transgene. From these data it is tempting to speculate that MeHg interferes with the Shh signaling pathway. (Figure 4.13 B, D).

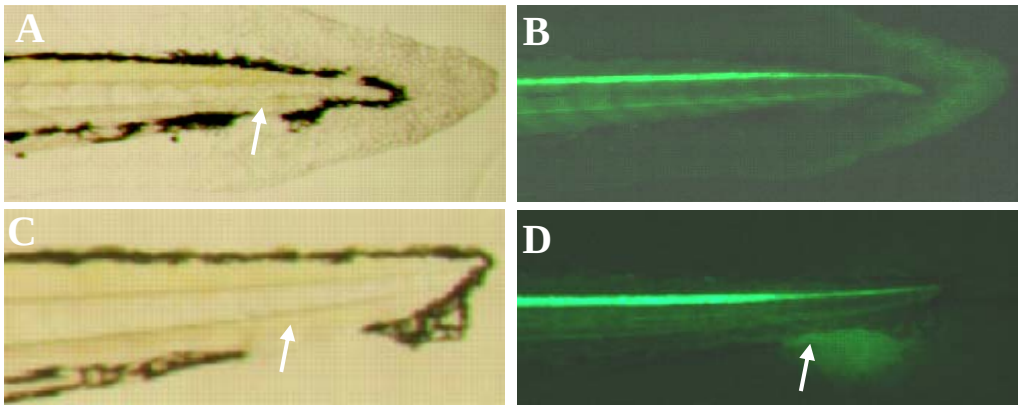


Figure 4.13 Morphological changes in tail fin pigmentation of embryos exposed to methylmercury from 4 to 48 hpf. A,C: The treated embryo (A) and control embryo (C) show a different tail fin shape and pigmentation. The embryos exposed to 50 µg/l methylmercury lack the gap in pigmentation characteristic of normal embryos (arrow). B,D: When the same embryos were observed under a fluorescence microscope, it is clear that the MeHg treatment abolished also the expression of the transgene *-2.4shh:gfpABC* line 15 (Arrow in B, D).

4.7.2 Methylmercury toxicity study

Matrix metalloproteinases (MMPs) are a subfamily of the large metzincin family of metalloendopeptidases characterized in part by their requirement for zinc. Currently, there are more than 20 characterized mammalian matrix metalloproteinases and numerous homologues in other organisms have been detected (Bosman et al., 2003). Matrix metalloproteinases were initially named according to their perceived substrate specificity. For example, MMP-1, 8 and 13 are also known as collagenase; MMP-2 and 9 as gelatinase; MMP-3, 10 and 11 as stromelysin; MMP-7 and 26 as matrylisin. Generally, it is accepted that MMPs in combination can degrade the entire extracellular matrix. MMPs play an essential role in many physiological events, including development, hormone dependent tissue remodeling, and tissue repair. They also play a key role in pathological conditions such as inflammation, tumor invasion, and metastasis. Most of the MMPs are secreted proteins that typically contain a secretory signal sequence, a pro domain that maintains the zymogen status, and a catalytic domain that contains a zinc binding site consensus sequence.

4.7.2.1 Matrix metalloproteinase 9

In mammals, *MMP9* is mainly expressed in neutrophils and eosinophils, where it serves as a key marker for their differentiation (Dahlen et al., 1999). In zebrafish, *MMP9* transcription was first detected in unfertilized eggs, indicating a maternal contribution. In 12 hpf embryos, the expression domain of *MMP9* localized to the posterior region of the developing notochord. By 16-somites (17 hpf) *MMP9* expression has vanished in the anterior mesoderm of the embryo. By 24 hpf, discrete *MMP9* positive cells appeared to be present in the coalescing circulatory system of the embryo and become concentrated around the anterior yolk sac and the head of the embryo and in single cells along the length of the tail in circulation, similar to that observed with other myeloid markers. During the 48 to 72 hpf period, the expression of *MMP9* remained concentrated in macrophages, which are highly motile, large, round cells with cytoplasmic phagosomes that act as a bacterial defense mechanism, in the head region around the eye and to a lesser extent in the tail of embryo. In adult zebrafish, strong *MMP9* expression was found in the spleen (Yoong et al., 2006).

Interestingly, the additional *MMP9* expression domains were detected after zebrafish embryo was treated either from 4 to 24 hpf or from 4 to 48 hpf or from 4 to 72 hpf with 60 µg/l MeHg. In embryos exposed between 4 and 24 hpf, the expression of *MMP9* appeared to be increased in the skin around the whole body (Figure 4.14 B). By 48 hpf, *MMP9* expression had vanished in the skin but it was increased in the finfold of the trunk and tail (Figure 4.14 D). In embryos treated between 4 and 72 hpf, the expression of *MMP9* was detected in individual cells presumably macrophages and the strong level of transcripts along the dorsal and ventral fin especially in the tail was maintained (Figure 4.14 F). When observed at higher optical resolution, several *MMP9* expression areas are detectable around the tail fin. First, the *MMP9* mRNA was evident along the edge of the tail finfold. The second group of expressing cells accumulated at the end of tail. Finally, an interesting finding was that there was strong expression of *MMP9* mRNA in the three tail neuromasts, which are localized close to the gap of pigmentation in untreated embryos (Figure 4.14 G, H).

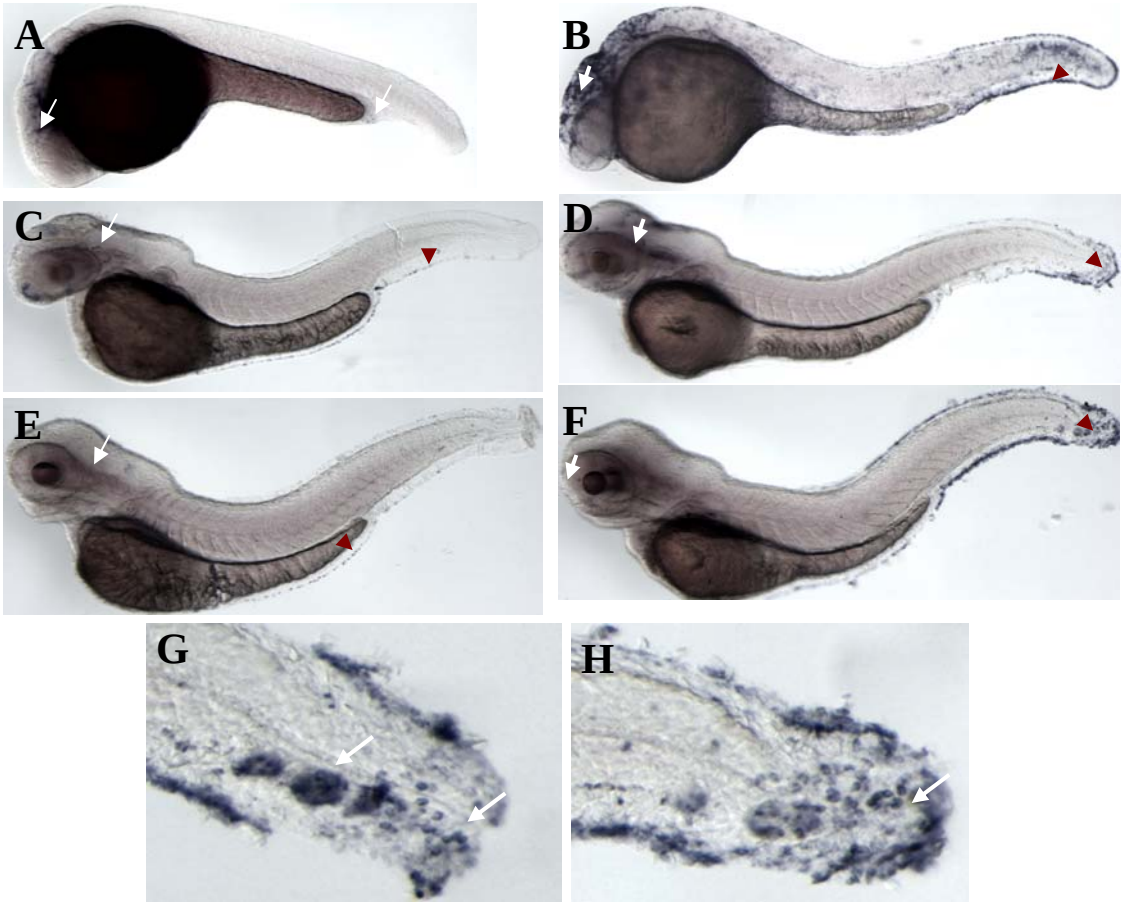


Figure 4.14 *MMP9* expression pattern in control and MeHg treated embryo. Staged embryos that are separated as control embryos (A, C, E) and embryos treated with 50 µg/l MeHg either from 4 to 24 hpf (B) or 4 to 48 hpf (D) or 4 to 72 hpf (F, G, H) were analyzed using a DIG-labeled *MMP9* probe (sites of *MMP9* expression shown with arrows). All the embryos are orientated in a lateral view with the anterior to the left and the dorsal side up and *MMP9* expression indicated with arrows. G, H: High magnification tail fin of the treated embryos, *MMP9* expression indicated with arrows. Three neuromasts (G, arrow) between the tail fin gap and some other cells around tail fin were induced to express *MMP9* mRNA (H, arrow).

4.7.2.2 Matrix metalloproteinase 13

A point mutation in MMP13 causes the Missouri variant of spondyloepimetaphyseal dysplasia, a human syndrome with abnormailities in development and growth of endochondral skeletal elements (Kennedy et al., 2003). *MMP13* is expressed by both terminal hypertrophic chondrocytes and osteoblasts, and its substrates include both Col1

and Col2. Furthermore, *MMP13* can synergize with *MMP9* in degradation of collagen (Engsig et al., 2000). The two major components of the extracellular matrix of cartilage, collagen type II and aggrecan, were studied as in vitro substrates for MMP13. The degradation of collagen type II and aggrecan is a coordinated process, in which MMP13 works synergistically with MMP9. Mice lacking both MMP13 and MMP9 had severely impaired endochondral bone, characterized by diminished ECM remodeling, prolonged chondrocyte survival, delayed vascular recruitment and defective trabecular bone formation (resulting in drastically shortened bones) (Stichens et al., 2004).

I first investigated the expression pattern of *MMP13* in normal zebrafish embryos. At 24 hpf embryos, discrete *MMP13* positive cells appeared to be present in the circulatory system of the embryo and were concentrated around the anterior yolk sac (Figure 4.15 A, B). By 48 hpf, the expression of *MMP13* was revealed as positive cells around the eye and at the posterior yolk extension (Figure 4.15 E). At 48 hpf embryo, *MMP13* transcription was found at the ventral head and in the circulatory system (Figure 4.15 G).

Embryos exposed to MeHg either from 4 to 24 hpf or from 4 to 48 hpf or from 4 to 72 hpf had a drastically increased expression of *MMP13*. In embryos treated from 4 to 24 hpf, the number of *MMP13* expressing cells was increased (Figure 4.15 C, D). Embryos treated until 48 hpf had increased expression of *MMP13* mRNA along the fin folds and around the eye. A lot of positive cells were concentrated at the ventral and posterior aspects of the eye (Figure 4.15 F). In embryos treated until 72 hpf, the expression of *MMP13* still remains in macrophages, which appeared in the circulatory system and concentrated around the eye. Furthermore, *MMP13* expression was strongly increased along the tail fin (Figure 4.15 H).

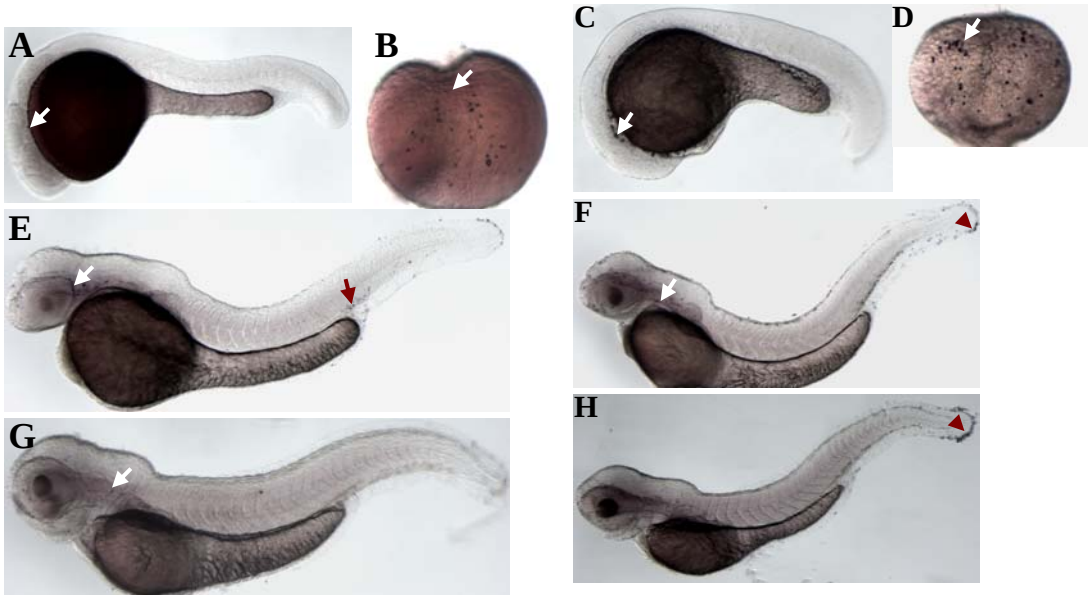


Figure 4.15 *MMP13* expression pattern Whole mount in situ hybridization. Staged zebrafish embryos that are separated as control embryos (A, B, E, G) and embryos treated with 50 µg/l MeHg either from 4 to 24 hpf (C, D) or 4 to 48 hpf (F) or 4 to 72 hpf (H) were analyzed using a DIG-labeled *MMP13* probe. The sites of *MMP13* expression are shown with arrows. In (A, C, and E-F) embryos are orientated in a lateral view with the anterior to the left and the dorsal side up. The embryos in (B and D) are orientated in a dorsal view with the anterior up.

4.7.2.3 *MMP9* and *MMP13* double in situ hybridization

The previous studies suggested that *MMP9* and *MMP13* are expressed in macrophages. I wished to assess whether the two genes are indeed co-expressed and carried out double in situ hybridization. In 48 hpf control embryos, *MMP13* expression was detectable in cells, which localized around the eye and in the ventral tail (Fig. 4. 16 A, C blue). In comparison to *MMP13*, fewer cells expressed *MMP9* and the expression of *MMP9* was restricted to cells in the circulation system (Figure 4.16 C).

In 48 hpf embryos treated with MeHg, the *MMP13*-positive cells were found around the eye and along the vasculature. Furthermore, some *MMP13* expressing cells were localized in the fin fold or in the tail immediately adjacent to the fin fold (Figure 4. 16 B, D, E, F). However, the cells that expressed *MMP9* mRNA localize to the tail fin. These

MMP9 positive cells (red) were different from the *MMP13* positive cells (Figure 4. 16 E, F). Taken together *MMP9* and *MMP13* appear to be expressed in distinct populations of cells. The identity of these cells needs to be determined in detail by additional experiment which was beyond the scope of this work.

After exposure of the embryos to MeHg until 72 hpf, the number of cells that expressed *MMP13* and *MMP9* mRNA were drastically increased (Figure 4. 17 B, D, F, G, H). These results supported the microarray data. Further this shows that the *MMP9* and *MMP13* genes are induced in a highly restricted pattern in embryos.

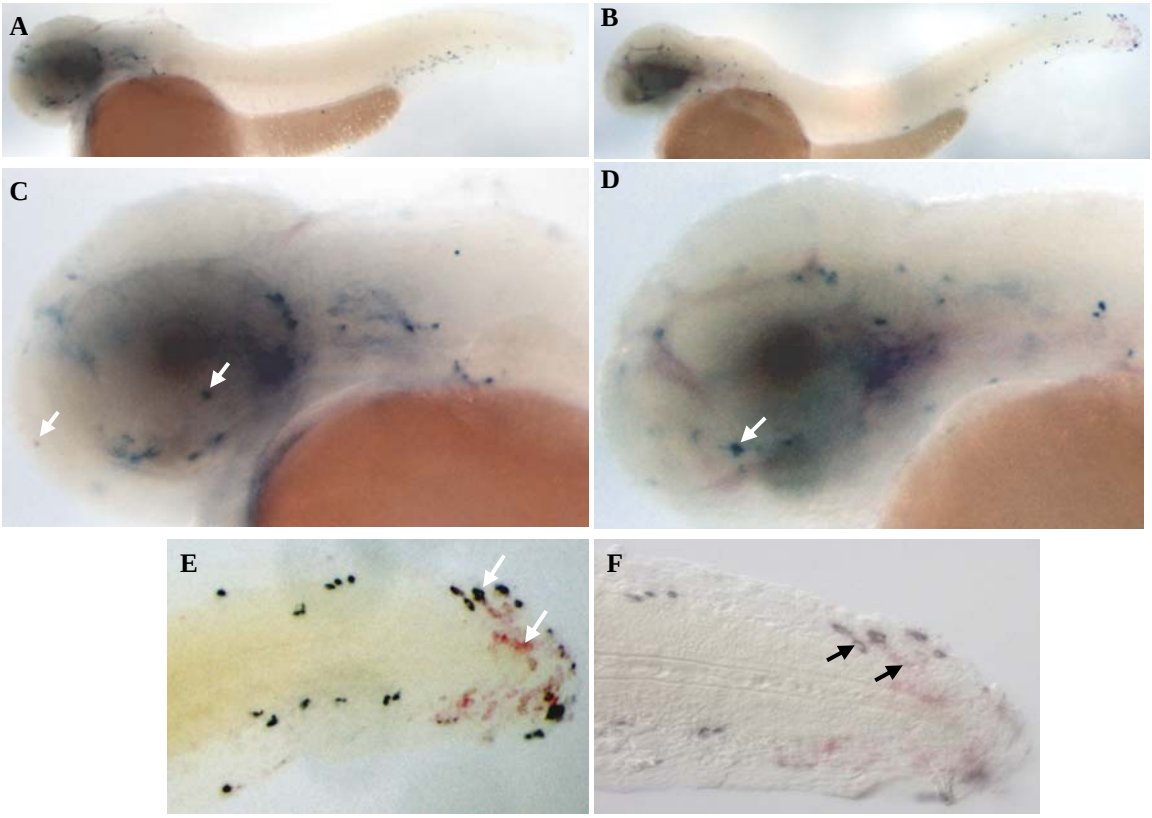


Figure 4.16 *MMP9* and *MMP13* expression pattern. Embryos at the indicated stages that are separated as control (A, C) and treated with 50 µg/l MeHg from 4 to 48 hpf (B, D, E, F) were hybridized with fluorescein labeled *MMP9* antisense probe and digoxygenin labeled *MMP13* antisense probe. All the embryos are orientated in a lateral view with the anterior to the left and dorsal up. *MMP9* and *MMP13* expressing cells are indicated with arrows.

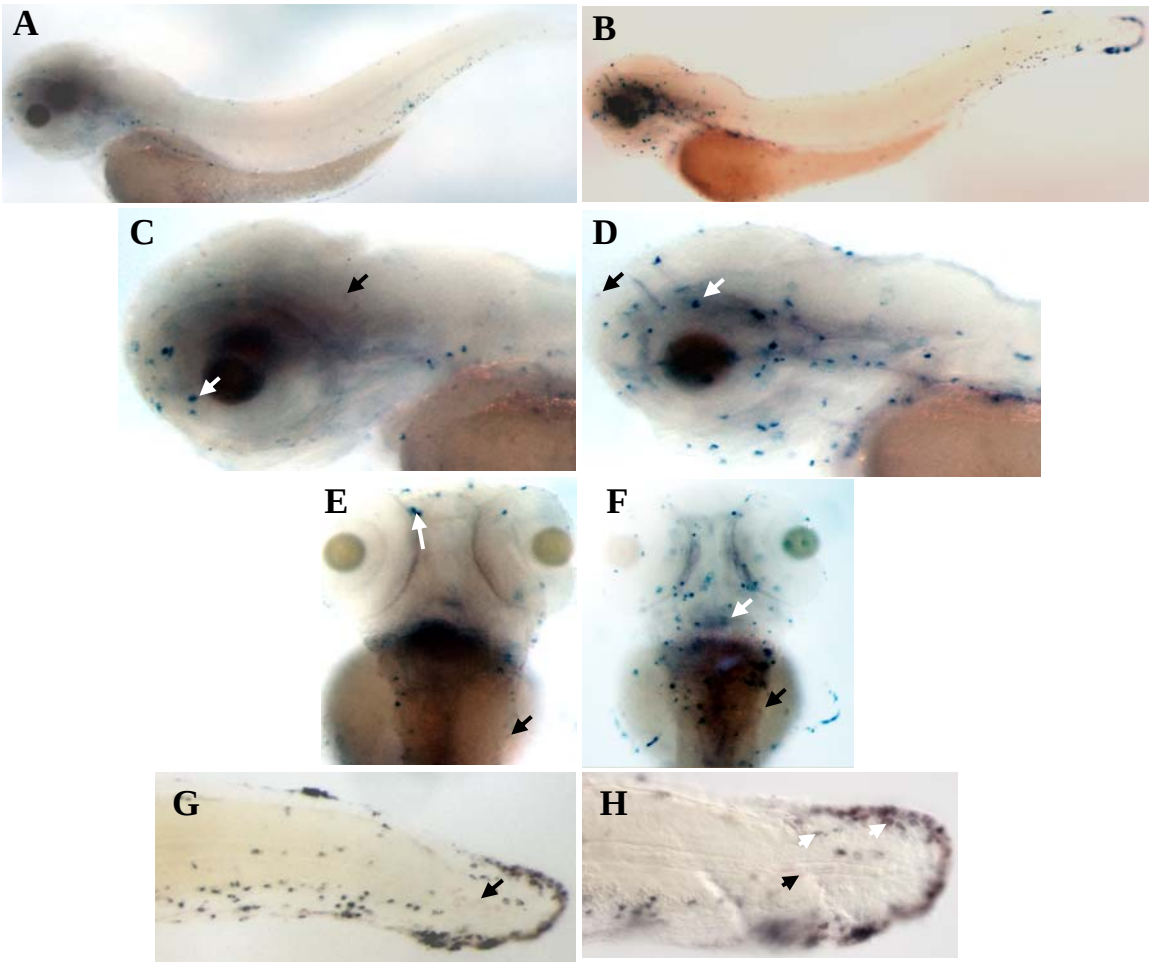


Figure 4.17 *MMP9* and *MMP13* expression pattern. Whole mount in situ hybridization analysis. Control embryos (A, C, E) and embryos treated with 50 µg/l MeHg from 4 to 72 hpf (B, D, F, G, H) were hybridized with fluorescence labeled *MMP9* antisense probe and digoxygenin labeled *MMP13* antisense probe. In (A, B, C, D, G, H) embryos are orientated in lateral view with the anterior to the left and the dorsal side up, *MMP9* expression is indicated with black arrows and *MMP13* expression indicated with white arrows. In (E, F) embryos are orientated in a dorsal view with anterior is up.

4.7.3 Cadmium toxicity study

A previous study in zebrafish revealed that cadmium exposure leads to localized cell death, alters muscle and motoneuron development and induces stress responses (Chow et al., 2003). I showed here that cadmium alters the expression of a number of genes that may play a role in the response of the embryos to Cd exposure. To elucidate the mechanism of cadmium responses of the embryos, several Cd-regulated genes, like

MMP9, MMP13, Thioredoxin and *parvalbumin 3a* were focused on key studying the expression patterns by in situ hybridization.

4.7.3.1 Matrix metalloproteinase 9 (*MMP9*)

The expression of *MMP9* is altered in embryos treated with cadmium from 4 to 24 hpf, from 4 to 48 hpf and from 4 to 72 hpf (Figure 4.18). By the 4 to 24 hpf exposure, the expression of *MMP9* was induced in the skin and macrophages (Figure 4.18 B). At 48 hpf, the induction of *MMP9* was concentrated in the margin of the tail finfolds besides the expression in macrophages (Figure 4.18 D). By 72 hpf, the majority of *MMP9* mRNA was detected along the side of tail fin (Figure 4.18 F).

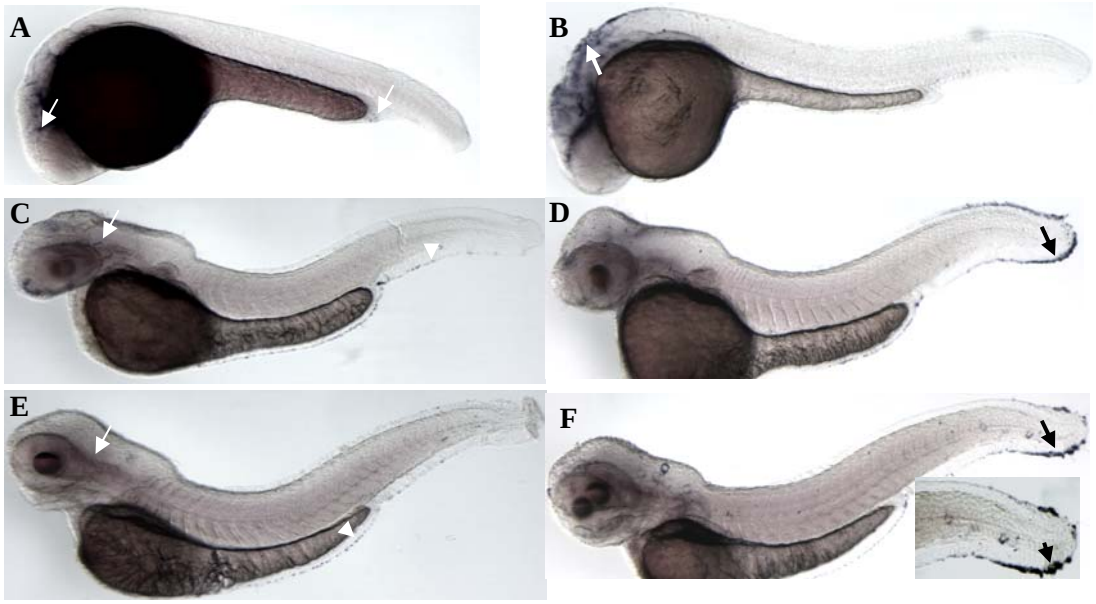


Figure 4.18 *MMP9* expression in embryos treated with Cd. Staged embryos that are separated as control embryos (A, C, E) and embryos treated with 500 µg/l cadmium either from 4 to 24 hpf (B) or 4 to 48 hpf (D) or 4 to 72 hpf (F) were analyzed using a DIG-labeled *MMP9* probe. All the embryos are orientated in a lateral view with the anterior to the left and the dorsal side up and *MMP9* expression indicated with arrows.

4.7.3.2 Matrix metalloproteinase 13 (*MMP13*)

The transcription level of *MMP 13* regulated by exposure of embryo to cadmium either from 4 to 24 hpf or from 4 to 48 hpf or from 4 to 72 hpf was revealed in Figure 4.19. At

24 hpf exposure, the number of *MMP 13* positive macrophages was higher than in control embryo (Figure 4.19 A, B, C, D). In addition, a group of positive cell was detected in the end of the yolk extension of exposed embryos. After 48 and 72 hpf exposure, the expression of *MMP13* was detectable in the vasculature but the majority of positive cells can be found around the eyes especially, at the posterior side of the eyes (Figure 4.19 E, F, G, H)

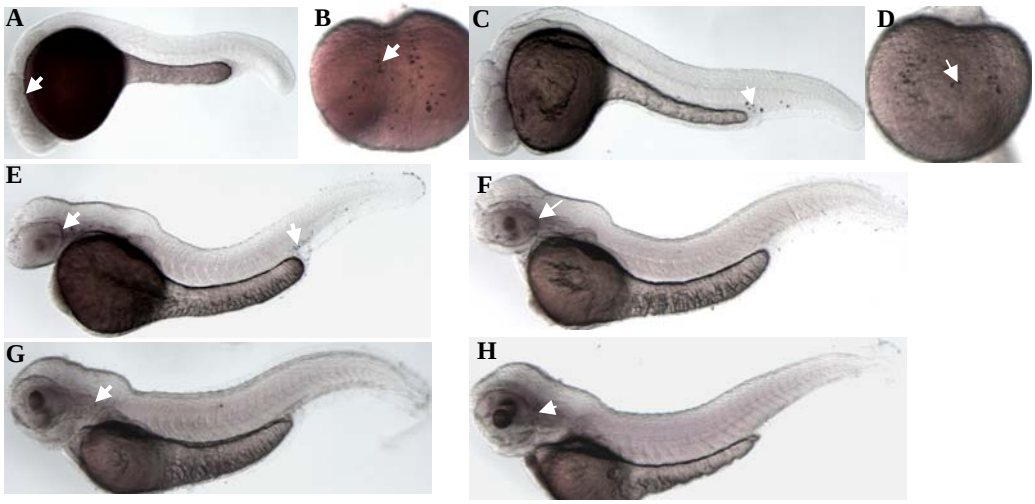


Figure 4.19 *MMP13* expression pattern in embryos treated with Cd. Staged control embryos (A, B, E, G) and embryos treated with 500 µg/l cadmium either from 4 to 24 hpf (C, D) or 4 to 48 hpf (F) or 4 to 72 hpf (H) were analyzed using a DIG-labeled *MMP13* probe. The embryos are orientated in a lateral view with the anterior to the left and dorsal up and mmp13 expression indicated with arrows (A, C, E, F, G, H). The embryos (B, D) are in dorsal view with the anterior up and *MMP13* positive cells indicated with arrows.

4.7.3.3 Parvalbumin 3a

The lateral line is a sensory system in fish and amphibians and responds to changes in the motion of water. It is involved in a large variety of behaviors, from prey detection to predator avoidance, school swimming and sexual courtship. It has disappeared in terrestrial tetrapods, with the exception of its internal counterpart, the inner ear. The lateral line comprises a set of sensory organs, the neuromasts (Figure 4.20) arranged on the body surface in species-specific patterns. Neuromasts comprise a core of mechanosensory hair cells, surrounded by support cells, and are innervated by sensory neurons that are localized in cranial ganglia called anterior and posterior lateral line

ganglia. The neuromasts on the head form the so-called anterior lateral line system, the ganglion of which is located between the ear and the eye, while the neuromasts on the body and tail, including those on the caudal fin, form the posterior lateral line system. Its ganglion is just posterior to the ear (Ghysen, et al., 2004). The neuromasts of the posterior lateral line are deposited at regular intervals by a primordium, which originates from a placode that is just posterior to the otic placode, and migrates along the horizontal myoseptum. The path followed by the primordium is defined by the expression of a zebrafish homolog of the chemokine stromal-derived factor 1 (SDF1), which the primordium detects through the expression of its receptor Cxcr4b. Knockdown of either the ligand or the receptor results in a similar strong defect in migration of the posterior lateral line primordium (Davie et al., 2002; Li et al., 2004).

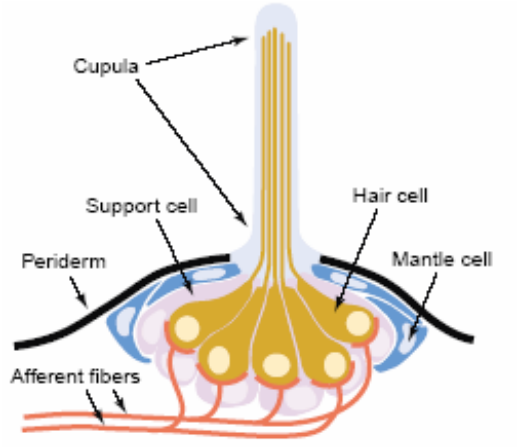


Figure 4.20 Scheme of a neuromast illustrating the different cell types. The cupula is secreted by the support cells

The mechanosensory hair cells of the inner ear sensory epithelia are commonly lost through aging, as well as noise- and drug-induced trauma. In particular, mechanoreceptive hair cells are extremely sensitive to ototoxicity, like aminoglycoside antibiotics, including neomycin (Harris et al., 2003). Recent studies revealed that the heavy metal copper can trigger the death of hair cells in the neuromasts of exposed zebrafish larvae (Hernardes et al., 2006; Linbo et al., 2006). While the exact mechanisms

of ototoxicity remain unknown, it appears to involve both known apoptotic cell death pathways and the formation of free radicals.

Given the fact that copper ions can induce loss of hair cells and the observation that a number of genes are specifcally expressed and induced in the neuromasts, I investigated whether neuromasts are present in Cd treated embryos. The fluorescent dye 2-[4-(dimethylamino)styryl]-N-ethylpyridinium iodide (DASPEI; molecular probes, Eugene, OR) is a dye to stain specifically hair cells in neuromasts of living embryos. The embryos were incubated in fish medium containing 0.005% DASPEI for 20 min and anesthetized in 10 µg/ml methanesulfonate salt, rinsed in fresh fish medium and then analyzed under a fluorescence microscope. The hair cells in the neuromasts were strongly stained with DASPEI in control embryos (Figure 4.21 A). The hair cells revealing high fluorescence intensity can be separated into two groups. One group is formed by the 18 anterior neuromasts around the eye; the other group of neuromasts is located along the horizontal myoseptum comprising 7-9 neuromasts of the posterior lateral line. The embryos treated with 500 µg/l cadmium from 4 to 96 hpf showed a decrease of fluorescence intensity in both anterior and posterior lateral line neuromasts. Interestingly, the neuromasts in treated embryos were deposited at the correct location (Figure 4.21 B). In other words, the number of hair cells in the anterior and posterior lateral line was decreased by cadmium exposure while the migration of the primordium depositing the neuromasts does not seem to be affected. To investigate the defect in the lateral line in more detail, I chose *parvalbumin 3a* and *thioredoxin* for further study.

Parvalbumin 3 is a prominent Ca^{2+} binding protein. Oncomodulin, the mammalian orthologue of parvalbumin 3 has been reported to be expressed in hair cells. In a previous study, parvalbumin 3 and related proteins have been suggested to be involved in the Ca^{2+} buffering capacity of different types of hair cells (Heller et al., 2002). According to this notion, Ca^{2+} signaling serves distinct purposes in different parts of a hair cell. The Ca^{2+} concentration in stereocilia regulates adaptation and, through rapid transduction channel reclosure, underlies amplification of mechanical signals. In the presynaptic active zones, Ca^{2+} mediates the exocytotic release of afferent neurotransmitters. At efferent synapses,

Ca^{2+} activates the K^{+} channels that dominate the inhibitory postsynaptic potential. A copious supply of diffusible Ca-binding protein buffer can isolate the three signals by restricting the spread of free Ca^{2+} and limiting the duration of its action.

The expression of *parvalbumin 3a* in embryos treated with cadmium revealed that the expression of *parvalbumin 3a* mRNA was reduced in both the anterior and posterior lateral line (Figure 4.21 C, D). The DASPEI staining showed that the number of hair cells in neuromasts was decreased drastically in Cd-treated embryos. The *parvalbumin 3a* mRNA level may then be reduced as a consequence of the less of hair cell. It remains to be tested whether the putative Ca^{2+} buffering protein parvalbumin 3a could be the direct trigger of Cd toxicity in hair cell.

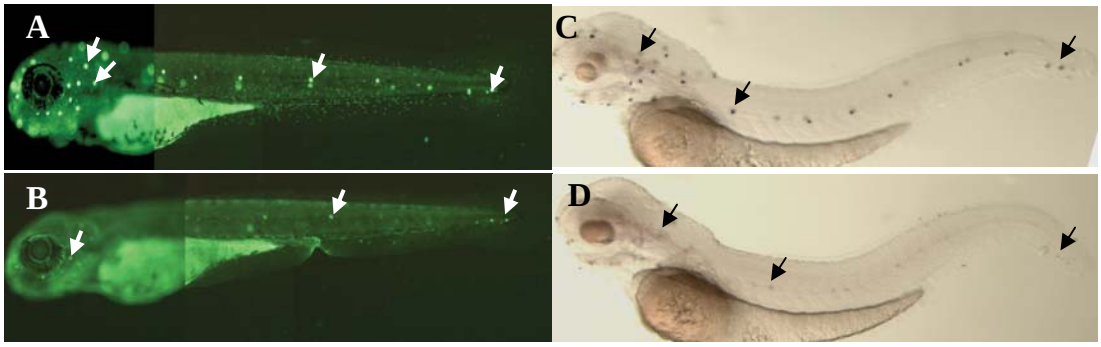


Figure 4.21 The embryos were stained with DASPEI and hybridized with DIG labeled anti *parvalbumin 3a* probe. Embryo were treated with 500 µg/l cadmium from 4 to 96 hpf (B) or the vehicle control (A) were stained with 0.005% DASPEI (2-[4-(dimethylamino)styryl]-N-ethylpyridinium iodide) in fish medium for 20 min, anesthetized and rinsed in fresh fish medium and then analyzed under a fluorescence microscope. The embryos exposed to 500 µg/l cadmium from 4 to 72 hpf (D) or control embryos (C) were fixed and hybridized with the anti *parvalbumin 3a* (or *oncomodulin A*) probe. The fluorescein stained hair cells in the neuromasts and the expression of parvalbumin mRNA are indicated by arrows

4.7.4 Thioredoxin

Thioredoxin, a small, ubiquitous thiol [sulfydryl(-SH)] protein, is one of the most important regulators of redox balance and redox-controlled cell functions. The biological

properties of thioredoxin rely, largely, on their reduction oxidation activity, which is the ability to transfer reducing equivalents to disulfide groups in target proteins. The key to the redox activity of thioredoxin is the presence of two cysteine residues separated by two amino acids in its active site. These cysteines exist as dithiols [-(SH_2)] in the reduced form and a disulfide (-S_2) in the oxidized form. Thioredoxin is oxidized when it transfers reducing equivalents to disulfide groups in target proteins and it is reduced back to the dithiol form by an NADPH-dependent flavoprotein, thioredoxin reductase (Burke-Gaffney et al., 2006). In humans, multiple thioredoxins exist: thioredoxin 1, thioredoxin 2 and thioredoxin-like molecules.

The biological activities of thioredoxin can be categorized as antioxidant, growth promoting, anti-apoptotic and inflammation modulating. The principle intracellular antioxidant property of thioredoxin results from its ability to act as a cofactor that maintains several thioredoxin peroxidase enzymes in a reduced, active form (Watson et al., 2004). Mechanisms of thioredoxin induced growth are multifaceted and include activation of transcription factors and increasing sensitivity of cells to cytokines and growth factors. At least 64 redox-regulated transcription factors have been identified. A number of these have critical thiol moieties and are known to be regulated, at least in part, by the thioredoxin system. Of particular note are p53, NF-κB, AP-1, Nrf2, GR, and ER, each of which is thiol dependent and has been implicated in processes such as cell proliferation and apoptosis. Thioredoxin's effects on apoptosis might relate to its ability to bind to and inhibit apoptosis signal regulating kinase 1 (ASK1). Thioredoxin overexpression provides protection against oxidative stress and reduces the induced toxicity of certain xenobiotics (Chen et al., 2002).

The glutathione and thioredoxin systems appear to act in parallel sharing the task to control the redox balance in the cell. Glutathione is present at millimolar concentrations in cells and functions along with several peroxidases and glutathione S-transferases to provide a primary protection against ROS and electrophiles. In addition, glutathione can reduce protein disulfides and sulfenic acids by nonenzymatic and enzymatic reactions.

The redox state of the glutathione and thioredoxin systems appears to be independently controlled. Thioredoxin is maintained in a reduced state, even under conditions resulting in glutathione depletion and oxidation (Nkabyo et al., 2002).

Cd induces a homolog to mammalian thioredoxins strongly. To date, nothing is known about thioredoxins in zebrafish. I thus investigated the pattern of expression of this thioredoxin in detail by using whole mount in situ hybridization. At 20 hpf, the expression of *thioredoxin* is detected in neural cells of the trunk forming two lateral rows of scattered cells (Figure 4.22 A, B). The distribution and shape of the cells suggests that these cells are a subclass of ventral interneurones. This expression in the neural tube of the trunk vanishes and at later stages a different pattern of expression is observed. The expression pattern of *thioredoxin* at 48 hpf is found in discrete groups of cells at the midbrain/hindbrain boundary**,** ventral thalamus, and olfactory epithelia. In addition, individual positive cells can be found in the hindbrain (Figure 4.22 C, D). In summary the expression pattern suggests that this thioredoxin like protein has very special activities in a restricted number of neuronal cells. In addition the expression appears to be modulated during development.

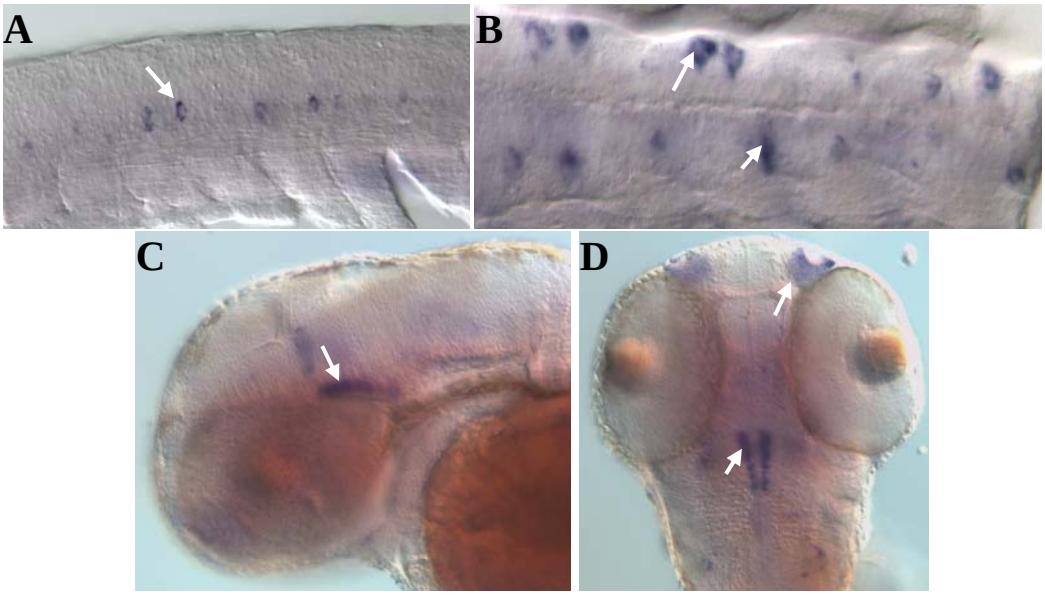


Figure 4.22 The embryos hybridized with DIG labeled anti *thioredoxin* probe. Staged zebrafish embryos (A, B(20 hpf), C, D(48 hpf)) were analyzed using a DIG-labeled anti *thioredoxin* probe. The embryos are orientated in a lateral view with the anterior to the left and the dorsal side up and *thioredoxin* expression indicated with arrows (A, C). The embryos (B, D) are in dorsal view with the anterior to the up.

According to my microarray data, *thioredoxin* mRNA transcription was up-regulated drastically in embryos exposed to Cd. Moreover, in addition to Cd as inducer, *thioredoxin* (BI864190) transcription levels were up-regulated by several other chemicals including As, 4CA, MeHg, Pb, PCB, and tBHQ suggesting that thioredoxin induction is a general response to a variety of toxins.

Next, I investigated whether the pattern of *thioredoxin* expression is changed in response to MeHg or whether the changes in levels are due to an up-regulation only in the expressing cells? The expression of *thioredoxin* was analysed in embryos treated with MeHg from 96 to 120 hfp followed by in situ hybridization (Figure 4.23). In 120 hpf control embryos, the expression of *thioredoxin* was detected in the olfactory epithelia, ventral thalamus and several positive cell groups around the hindbrain (Figure 4.23 A). The expression of *thioredoxin* mRNA was up-regulated dramatically in the olfactory

epithelia and ventral thalamus in MeHg treated embryos (20/20 exposur embryos)**.** Surprisingly, several additional expression domains were detected: The anterior and posterior lateral line, cell groups in the hindbrain, the epithelia of mouth and swimming bladder expressed the gene in MeHg treated embryo. (Figure 4.23 B, C, D, E).

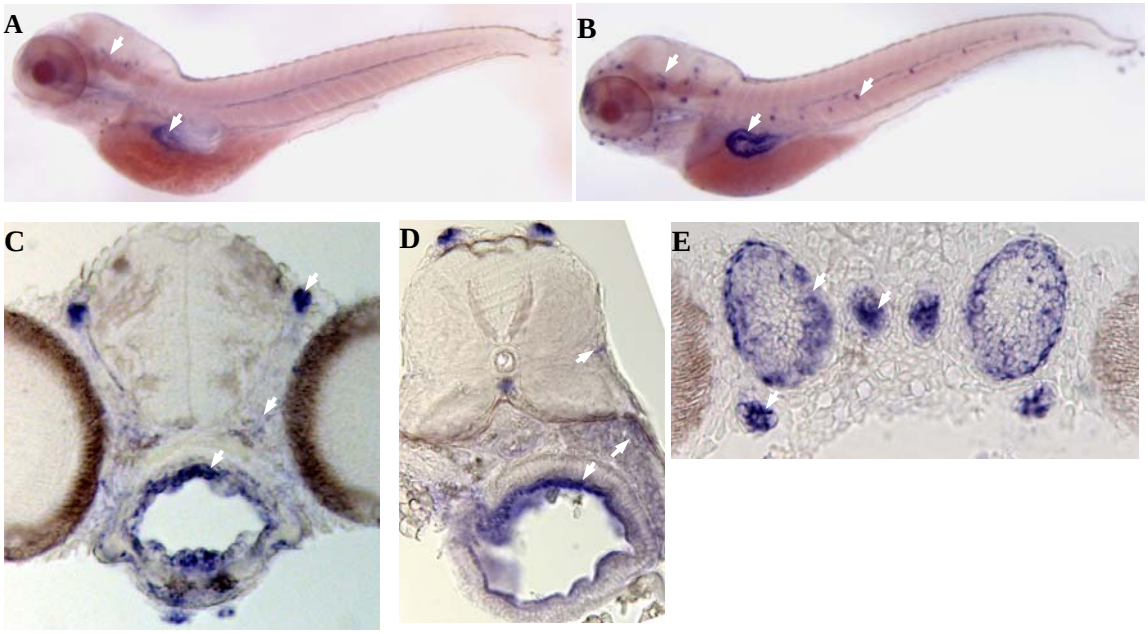


Figure 4.23 Thioredoxin expression pattern in 120 hpf MeHg treated embryos (B, C, D, E) and controls (A). The 120 hpf control embryos (A) or treated with 60 µg/l MeHg from 96 to 120 hpf were fixed and hybridized with DIG labeled anti thioredoxin probe. Arrows indicate the thioredoxin expression. Lateral views (A, B) and transverse sections (C, D, E) of treated embryos (20/20 treatment embryos).

In the dorsal view of a lateral line neuromast, the positive cells were arranged at the boundary of the neuromast and the skin epithelia around the hair cells in the middle of the neuromasts (Figure 4.24 D). The expression of *eya 1* and *parvalbumin 3a* was visualized by two-color in situ hybridization. In the dorsal and lateral view, the mRNAs of *eya 1* and *parvalbumin 3a* were detected in both hair cells and supporting cells (Figure 4.24 B, E). But it is not clear whether the two genes are expressed in mantel cells.

The expression of *thioredoxin* mRNA was induced in the anterior and posterior lateral line after exposure of embryos to Cd from 4 to 72 hpf. As shown by Daspei staining (Figure 4.21) the number of hair cells is reduced in Cd-treated embryos. The *thioredoxin*

mRNA was strongly expressed in the remaining hair cells in Cd treated embryos. (Figure 4.24 C, F). It remains to be established whether the hair cells that were induced to express *thioredoxin* mRNA by Cd exposure are cells that survived the impact of Cd or whether these are newborne hair cells in an attempt to regenerate the neuromast.

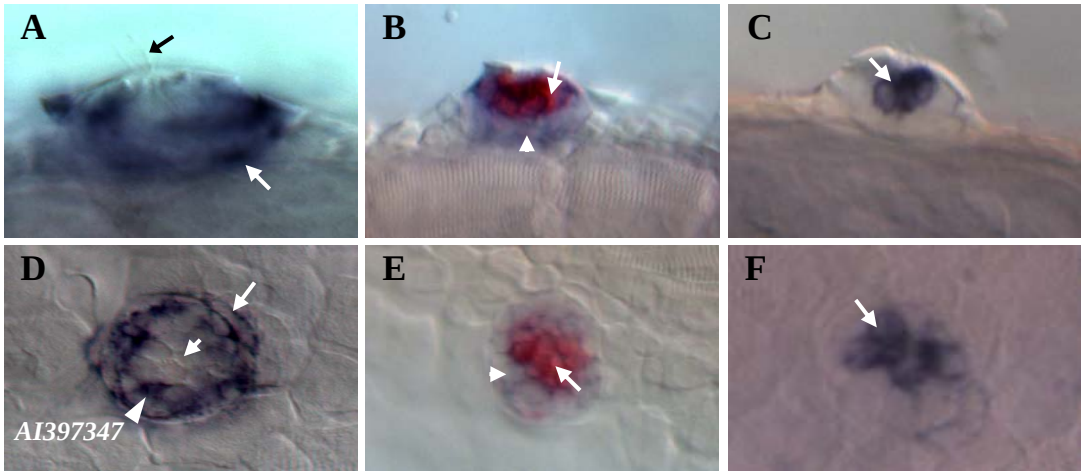


Figure 4.24 Gene expression pattern in neuromast. The staged embryos were fixed and hybridized with dig labeled anti (AI397347, panel A, D), anti *parvalbumin 3a* (panel B, E) and anti *thioredoxin* (panel C, F) or with fluorescein labeled anti *eya 1* (panel B, E) RNA probe. At 72 hpf control embryos (A, B, D, E) or treated with cadmium from 4 to 72 hpf (D, F) embryos were revealed three genes expression patterns in neuromasts. A, D showed gene *AI397347*, arrows, arrowhead, short-arrow, and black arrow indicate the mantle cells, the support cells, hair cells, and the cilia respectively. B, E showed *eya 1* (red) and *parvalbumin 3a* (blue), arrows and arrowhead indicate the hair cells and support cells. C, F showed *thioredoxin*, arrows indicate the hair cells. Embryos are oriented anterior to the left and dorsal up (A-C) or view onto dorsal (D-F).

5 Discussion

In summary, this thesis work reveals that: 1) Stage-specific toxicogenomic responses. 2) The toxicogenomic responses triggered by different toxicants are highly specific. 3) Toxicants induced tissue specific genes expression. 4) The genomic responses are very sensitivity to toxicants insult. 5) Complex synergy effects are evident in toxicogenomic responses to compound mixtures.

5.1 Traditional approachs for assessing toxicity

For their many advantages as model organism, zebrafish have been used in a variety of applications to assess environmental toxicity. For example, zebrafish assays have been used to directly monitor water, soil, and waste water quality for ecotoxicity studies (Ruoppa et al., 1988; Vitozzi et al., 1991). Furthermore, zebrafish have also been used to assess risks of toxicity, to predict toxicity associated with petroleum products and by-products, to evaluate working conditions in the petrochemical and mining industries, and to study the effects of polychlorinated biphenyls, dioxin and other toxicants (Lele et al., 1996; Andersson et al., 2001; Andreasen et al., 2002; Ton et al., 2006). Moreover, a number of guidelines from the Organization of Economic Cooperation and Development (OECD) recommend zebrafish as a model for aquatic toxicity testing. In the DarT (Danio rerio embryo toxicity test. Nagel R., 2002), embryos are briefly (48 hours) exposed to the test substance, followed by analysis of several parameters, such as somite formation, development of organs, pigmentation, edema formation, tail length, heartbeat and movement. This assay is considered as a pain-free *in vitro* test and is therefore accepted as a replacement for animal experiments in Germany. DarT correlates well with the acute fish toxicity tests but it has low sensitivity for assessment of low dose exposure effects. Other readouts need to be developed to assess the adverse effects of low dose exposure.

5.2 Gene expression profile and toxicogenomics

In the last decade, a new paradigm has emerged among toxicologist which is based on the assumption that by knowing the molecular mechanism of toxicity of compounds one

would be able to predict the risk emanating from novel compounds. Understanding the mechanism of action of toxicants in living organism will help to develop predictive simulation models of toxic effects to link molecular biomarkers with population level effects, and then to anticipate the ecologic risk for new chemicals. In fact, gene expression is a unique way of characterizing how cells and organisms adapt to changes in the external environment. The measurements of gene expression levels upon exposure to a chemical can be used both to provide information about the mechanism of action of the toxicant and to form a sort of genetic signature for the identification of toxic products. Toxicogenomics uses global gene expression analyses to detect expression changes that influence, predict or help define, and classify chemicals' toxicity. Especially, it is believed that toxicogenomics could accelerate the detection of toxic liabilities by replacing long-term exposure experiments for the selection of clinical candidates. Thus pharmaceutical companies have started to build their own database in the hope of predicting the potential toxicity of compounds (Khor et al., 2006).

5.3 Exposure windows and chemical classification

The gene expression profiles indicated that the biological response to toxicants is varying at different life stages. From 4 to 24 hpf exposures, the number of response genes is less than that induced at later stages. Moreover, the gene expression profiles of embryos treated between 4 to 24 hpf are less characteristic than those of the later stages. These early stages appear thus to be unsuitable to classify chemicals. One hypothesis is that, embryos do not activate or repress genes as efficiently in response to toxicants at this stage because organ systems have not matured sufficiently. Surprisingly, at this stage, the gene expression profiles show much higher statistical variation. In other words, the gene induction varys between different experiments and there may be also a higher variability in the response of individual embryos within one experiment. Another explanation is thus this higher variation in gene responses, which will lead to averaging out of specific gene responses. Moreover no data exist from mouse embryos at equivalent embryonic stages as these gastrula and blastula stages are very difficult to analyse in mammals. Thus comparative material is missing to support my conclusion.

Irrespective of the causes, my results indicate that the exposure window from 4 to 24 hpf is not useful to derive gene expression profiles complex enough for chemical classification. The other two exposure windows, from 24 to 48 hpf and from 96 to 120 hpf, in principle, can be used to study the gene expression profiles and classify the different toxicants.

The exposure window, from 96 to 120 hpf was chosen to study the gene expression profile and predict the toxicity for several reasons. At 96 hpf, the hatched larvae have completed most of the early morphogenesis and begin to swim. They also start to feed and hunt on the subsequent stages suggesting that they have an almost mature physiology and may thus have also more adult responses to toxicants. Experimentally, these older stages have the advantage that one needs to expose fewer animals to isolate sufficient mRNA for a microarray experiment.

The gene expression profiles of 11 toxicants were analysed by different statistical methods. Principal component analysis and hierarchical clustering of the gene responses induced by the 11 chemicals allowed separation of the toxicants by their gene response pattern. Comparison of the expression profiles of the 11 chemicals revealed three more closely related sub-groups: As, Pb, VA, Hg, Cd, and tBHQ belonged to the first group; TCDD, 4CA, DDT, and AA represent a second sub-group while PCB formed its own group.

Irrespective of these similarities, it was possible to identify toxicant-specific patterns. The verification of the toxicogenomic profiles in blind test experiments underscores this finding. Fourteen out of 15 chemicals were unambiguously identified. In the case of 4CA, similar Euclidean distances were found to DDT and AA response profiles thereby not allowing unambiguous identification of the chemicals. But, all three chemicals belong to the same subgroup. These results indicated that gene expression profiles can be used to classify different chemicals by their toxicogenomic profiles. This information may be useful to pick up signature for predicting the toxicity of unknown or novel chemicals.

In principle, the heavy metals may be expected to induce a similar response. Analysis of the induced gene ontologies suggests that Cd, As, Pb, and MeHg induce indeed similar responses. The transporter system genes such as *solute carrier family 16* (BE016639, BI474827), heat shock protein genes such as *Hsp70* (AB062116, AF210640, AF006007, except MeHg) and genes with ontologies related to transcription factors such as *ATF3* (AW422298), *Zinc fingers protein 1* (AI793802), and *C/EBP beta* (AW019436) were significantly up regulated by the heavy metals. In addition, genes encoding proteins involved in antioxidant responses (Winyard et al., 2005), for example, *peroxiredoxin 1* (BI980610), *thioredoxin like protein* (BI864190), and *glutathione S-transferase omega 1* (AW019036), and metalloproteinases such as *MMP9* (AW174507) and *MMP13* (AW305943) were triggered by heavy metals exposures. *6-phosphogluconate dehydrogenase* (AW11578), *Arginase type II* (BF717769, BG891938), and *ribosomal protein S17* (BI474700) genes were up regulated by most of these heavy metals. On the other hand, *GTP cyclohydrolase 1* (AJ311846), *Amylase alpha* (BM103972) *Keratin 6* (BE016992), and *Keratin 13* (BE200701) were down regulated by Cd and Pb exposures. Most of above response genes were found to be regulated by VA and tBHQ also. Interestingly, the Ca^{2+} buffering gene *parvalbumin 3a* (BE201681) was only responsive to heavy metal exposure and *cytochrome p450 1a* (AF057713), *solute carrier family 6* (BF157011, BI563084) were found in tBHQ and VA exposures respectively. Irrespective of these similarities of the first group, some specific response genes for each chemical can be found to identify the properties of the toxicants. For example, *glutathione peroxidase* (AW232474) was only repressed by MeHg; *heat shock protein 90-alhpa* (AF068773) was only triggered by As; *serum amyloid a* (BI883516) was induced by Cd; unknown gene (AI958489) was down regulated by Pb; *solute carrier family 6* (BF157011, BI563084) was specifically down regulated by VA.

A number of genes I found also showed to be induced by Cd, As, and Hg in other studies in mouse and zebrafish (Bartosiewicz et al., 2001; Lam et al., 2006; Liu et al., 2003), for example, *Hsp70*, *Hsp90 a*, *glutathione S-transferase Pi*, and *thioredoxin* etc. However, many new response genes were found to be induced specifically by heavy metals in my work. The reasons could be that the gene numbers on the respective microarrays were

different. For example, Bartosiewicz used 148 genes including xenobiotic metabolizing enzymes and only 9 genes were regulated by Cd in mice. A chip with 588 genes has been used in a Hg vapour exposure study and 23 genes were shown to be regulated (Liu et al., 2003). The same micoarray as ours was used to study the As toxicgenome of the adult zebrafish liver and more than 1700 genes were found to be regulated (Lam et al., 2006). These results are not comparable to those of the whole animal study used here. Furthermore, different concentrations, different forms of the chemicals, and different exposure times were used for these experiments, for example, 15 mg/l As (V) was used to expose adult fish for 24, 48, and 96 hours and 4 livers for each time point were analysed.

It has been shown that VA is a selective inhibitor of histone deacetylases and selectively reduces histone deacetylase 2 protein level (Gurvich et al., 2005; Menegola et al., 2006; Kawai et al., 2006). However my data do show that VA causes also the production of reactive oxygen species suggesting a further mechanism of teratogenesis by VA.

The monooxygenases were regulated significantly by TCDD, 4CA, DDT, AA. However, heat shock protein, transcription factors, and transporter related genes were not induced significantly by this group. The patterns of gene induction indicated that the 3 chemicals 4CA, DDT, and TCDD use the AHR/ARNT signaling pathway which regulates very specifically certain response genes, for example, *cytochrome P450 1a* (AF057713) was up regulated by the 3 chemcials. In addition, CA and TCDD induced *cytochrome p450 1c* (BG738243) while, *aryl hydrocarbon receptor 2* was triggered by TCDD. In fact, TCDD is a very specific toxicant for interacting with AHR. Several studies (Andreason et al., 2002, 2006; Handley-Goldstone et al., 2005; Bemis et al., 2007) have shown that the target genes, *CYP 1a1, CYP 1c1*, and *AHR* are specifically induced by TCDD. In regenerative growth of zebrafish, TCDD induced many genes including genes involved in, matrix metabolism, FGF signaling pathway, and many development related genes (Andreason et al., 2006). A study (Handley-Goldstone et al., 2005) with a zebrafish adult heart cDNA chip, showed that several sarcomeric genes, such as cardiac troponin T2, and genes involved in mitochondrial energy transfer were affected by TCDD.

Different experimental conditions could affect the response to toxicants. While, a large number of genes inducing xenobiotic metabolism related genes, metabolism related genes, and DNA damage and repairing related genes, were regulated by 4CA. The other members of subgroup 2, DDT, TCDD, and AA induced fewer genes and they form a group of chemicals within subgroup 2 with distinct transcription profiles. One reason could be that these chemicals act very specifically on a limited number of targets. In fact, the effects of TCDD may only be mediated by activation of the AHR/ARNT receptor complex (Andreason et al., 2002). On the other hand, the chemicals may have been applied at doses that are subacute. The latter is a critical issue for comparison of toxic effects and is not only dependent on the initial concentration but also on the penetrance and modification of a specific chemical in the embryo.

In fact, *CYP 1a1* was triggered by PCB but the induction was weaker than by TCDD. Previous studies have shown that PCBs and their hydroxylated metabolites interact with hormonal systems or through binding to AHR, induce AHR-dependent genes (Ulbrich et al., 2004) or cause alterations in intracellular free Ca^{2+} and affected second messengers (Kodavanti et al., 1993, 1994; Zawia NH., 2004). However, how PCBs do affect gene expression is still not clear. My results demonstrated that PCB exposure triggered changes in expression of genes from several different gene ontology groups, such as apoptosis (*Mcl-1a*), protein kinases (*MKK3, Jun protein 2*), genes involved in DNA repair (*APEX nuclease, MutS*) and antioxidants (*peroxiredoxin 1*, *thioredoxin*), transporters (*solute carrier family 16, 6*), heat shock proteins (*Hsp 70*), monooxygenases (*Cyp 1a1, Cyp 2a4*), metalloproteinases (*MMP9, MMP13*), oxidative stress and transcription related genes (*ATF 3*, *ATF 5*). In addition, metabolic enzymes such as *arginase type II*, *6-phosphogluconate dehydrogenase*, *delta-5 fatty acid desaturase*, *ribonucleotide reductase*, and *phosphoenolpyruvate carboxykinase 1* were induced strongly.

Interestingly, many cytoskeletal genes were down-regulated by the heavy metal group and PCB, for example, *keratin 6* and *keratin 13* were repressed by Cd and Pb; *keratin 25* was regulated by Pb, TCDD, and VA; *tubulin beta* was repressed by MeHg and VA.

Moreover, genes encoding metabolic enzymes were altered in the expression levels by most chemicals except DDT and TCDD, for example, *GTP cyclohydrolase 1* was down regulated by Cd, Pb, PCB, and tBHQ; *6-phosphogluconate dehydrogenase* was up regulated by As, CA, Hg, Pb, PCB, and tBHQ. These enzymes included energy metabolising enzymes, like *pyruvate dehydrogenase kinase*; protein metabolising enzymes, like *arginase*, and fatty acid metabolising enzymes, like *delta5/delta6 fatty acid desaturase*. This suggests that these chemicals disturb the normal metabolism of the embryo.

In previous studies (Blechinger et al., 2002; Krone et al., 2003), the Hsp70 gene has been promoted as a marker gene for heavy metal identification. However, it is not a unique response gene to heavy metal exposures as it can also be regulated by other toxicants. The expression of Hsp70 mRNA was not regulated significantly by MeHg even though some studies suggested Hsp70 expression can be induced by Hg exposure (Krone et al., 2003). My data suggest that Hsp70 related genes can be candidates as a signature gene for the first chemical group and PCB.

5.4 Low dose and synergistic effects

Specific gene expression profiles can be detected at low doses even though one cannot observe morphological defects. Thus expression profiles are more sensitive to detect the impact of chemicals than conventional endpoints. Toxicogenomic responses were triggered by TCDD, Cd, DDT, CA and VA at concentrations that did not exhibit changes in morphology. Thus the response of the genome appears to be more sensitive to toxic insult than morphogenesis. A crucial question is whether the gene responses that are not obviously correlated with pathological alterations are indeed deleterious to the animal. For example, TCDD was shown to induce a battery of genes in the mouse paw (including homologues of genes we scored in our study) without obvious teratological consequences to paw development (Bewis et al., 2007). Future work will need to address whether the low-level effects on gene expression could be correlated with and hence used to predict chronic effects of long term exposure.

The lowest concentration of MeHg (6 μg/l) triggered significant changes in gene expression. In addition, we noted also teratological effects on movement and tail development at these low concentrations (L.Y. and J.R.K., unpublished) indicating that these low concentrations of MeHg cause acute toxicity in the zebrafish embryo. Disturbingly, the blood serum levels of humans can be in the same concentration range http://www.cdc.gov/exposurereport/pdf/factsheet_mercury.pdf. The zebrafish embryo may be much more susceptible to MeHg. However, defining the blood serum levels that are regarded as save in humans, is an active area of research.

Contaminants are seldom present as a single chemical and usually comprise a complex mix. Application of a mixture of MeHg, Cd, As and Pb at low concentrations resulted in synergy effects with more than additive numbers of genes affected and also novel patterns of gene expression changes. Clearly, some of the genes affected by exposure to the mixture would be induced or repressed by higher concentrations of the individual chemical. Examples are *thioredoxin* and *hsp70*. Thus, it appears that the threshold, at which induction occurs, is lowered. This is congruent with previous studies of mixture effects that support the notion of "concentration addition", in which a component of the mixture can be replaced by an equipotent concentration of another compound (Escher et al., 2002). However, the patterns of gene expression changes induced individually by the 4 chemicals differed suggesting that other effects have to be taken also into account that can not be explained by an additive mechanism of action. For example, *glutathione S-transferase omega 1* and *Jun dimerization protein 2* (BI326453) induced by individual chemicals were not activated significantly by the mixture.

5.5 The mechanism of toxicity

Gene expression profiles represent a primary level of integration between environmental factors and the genome, which ultimately guides the response of an organism to external changes. Thus, the analysis of gene expression changes is a powerful tool both to diagnose major stressors and to analyze the mechanism of the stress responses. The early stress response genes induced by toxicants include transcription factors, heat shock

proteins and chaperones, and antioxidants activity related genes. Strikingly, Hsp70 related genes are often up-regulated by toxicants. In fact, HSP70 is the major inducible heat shock protein and is involved in many cellular activities, which include control of protein synthesis, folding and translocation, moreover HSP70 is also associated with protection against apoptosis with an inhibition of cytochrome c release from mitochondria (Water et al., 2006). HSP70 can prevent the onset of apoptosis caused by overexpression of apoptosis signal-related kinase1 (ASK1) (Park et al., 2002). In addition, HSP70 can bind directly to apoptosis protease activating factor1 (APAF1) which is essential in the formation of the apoptosome. Binding of HSP70 to the caspase activation recruitment domain of APAF1 prevents the formation of the apoptosome, and prevents activation of caspase 9 and 3 (Water et al., 2006).

Antioxidants related genes, such as thioredoxin, thioredoxin reductase, and glutathione S-transferase can adjust the activation of a kinase that is upstream of c-Jun N-terminal kinase (JNK), for example apoptosis signal-related kinase (ASK1) (Saitoh et al., 1998). In fact, JNK, p38, and extracellular signal-regulated kinases (ERKs) are all involved in stress signaling (Kyriakis et al., 2001) moreover the activation of JNK seems to involve an oxidative stress-related pathway.

Many antioxidant related genes are regulated significantly by the heavy metal group and PCB. In addition, several protein kinase genes were altered by PCB, for example, *MAP kinase interacting kinase 2* (BI533884), *MKK 3* (mitogen activated protein kinase kinase 3) (AB030889), *MMMNK1 map kinase interacting kinase* (BI705604), and *cell division control protein 2* (BI888928). Previous studies have shown that second messengers and hormone systems can be interfered with by exposure to PCB. Moreover, the down-stream signaling components, like AP-1 and NF-kB interact with different protein kinases. The gene induction profile demonstrated that AHR/ARNT signaling pathway and MAP kinase pathway were significantly induced by PCB. This could be the reason that many tissues, organs, especially the nervous system, were affected by PCB. The induction of CNS (central nerve system) defects by heavy metals, especially causing mental retardation and permanent cognitive deficits, is a major public health problem. Until now,

the mechanism of heavy metals leading to CNS deficits is not clear, but investigators suggested that these metals have the ability to substitute for calcium and zinc to cause many toxic effects (Lidsky et al., 2003; Zawia., 2004). In fact, the heat shock proteins and several transcription factors regulated by heavy metals, could be the cues to study the molecular mechanism.

Metalloproteinase and transcription activity related genes were also up regulated by this group. Several transcription factors, including ATF3 and C/EBP beta were induced by the toxicants (As, Cd, Hg, Pb, VA, PCB, and tBHQ). Strikingly, the induction of metalloproteinase expression was associated with the up-regulation of *ATF3* expression. In agreement, study suggested that the sequences of *Hsp70* and *MMP13* genes have ATF3 binding sites and the expression can be regulated by ATF3 (Okamoto et al., 2006). Misregulation of these metalloproteases may be part of the toxic mechanism of these chemicals.

The expression of *parvalbumin 3a*, which is a Ca^{2+} buffer in hair cells, was down-regulated specifically by heavy metal exposure. The mechanism of regulation of *parvalbumin 3a* is not known. It could be associated with an effect of heavy metal ions on Ca^{2+} homeostasis. Is there a relation of the reduction of the number of hair cells induced by heavy metals with the *parvalbumin3 a* or *thioredoxin* or other gene regulation? The hypothesis is that heavy metal ions change the ion balance of these cells and trigger expression or activity of transcription factors which regulate expression of these genes. To confirm this suggestion, further experiments need to be done. In particular, it may help to systematically analyse, which transcription factors are up or down regulated in the hair cells in response to toxicants.

Cytochrome p4501a1 is one of the xenobiotic metabolizing enzymes, which is induced by polyhydrogenated aromatic hydrocarbons via activation of the aryl hydrocarbon receptor signaling pathway. Although a great deal is known about AHR driven transcriptional regulation, much less is known about the mechanism by which TCDD causes toxicity and disease (Mandal PK., 2005). The expression of *Cyp4501a1* was up-

regulated significantly by the second group of toxicants especially by TCDD. Part of the toxicity of this group may thus be linked to AHR mediated signaling, even though some studies suggested that TCDD causes cellular effects that are independent of this signaling pathway (Tan et al., 2002). Besides the induction of *cyp4501a1* and *cyp4501b1*, several heat shock proteins and antioxidant-related genes were up-regulated by 4CA. Therefore, the toxicity of 4CA most likely involves other mechanism like induction of reactive oxygen species. In fact, the p45 NF-E2 related factor Nrf2, which is a basic leucine zipper family transcription factor involved in the regulation of antioxidant response element (ARE) mediated gene transcription, is believed to play an important role in detoxification by regulating many phase II detoxification enzymes (Voelker et al., 2007). Heat shock proteins and antioxidant related genes are main targets for Nrf2 (Kwak et al., 2003; Khor et al., 2006). A study suggested that transcriptional regulation of Nrf2 is mediated via activation of the AHR (Miao et al., 2005). However, the gene expression profiles revealed that the antioxidant and heat shock protein genes were not significantly regulated by TCDD and DDT. This suggests that these genes are induced by an AHR independent mechanism in the zebrafish embryo.

5.6 signature genes

Signature genes can be used as markers for identification of chemicals or groups of chemicals by comparing response patterns. In fact, a number of investigations define the suitability of CYP4501B1 for use as a mechanistically based biomarker in molecular epidemiology studies of human populations exposed to dioxins and related chemicals that bind AHR (Landi et al., 2003; Baccarelli et al., 2004). But the induction of *cyp4501b1* expression was found to be regulated by 4CA also in gene expression profile. Based on the expression profiles, I think, glutathione peroxidase gene should be a candidate as a biomarker for identification MeHg, while solute carrier family 6 (SLC6) gene can be as a biomarker of VA. The parvalbumin 3a gene can be a candidate for assessment heavy metal toxicity.

Although, gene expression profiling of *in vitro* system, like cell lines, is a useful tool for understanding the mechanism through which a compound exerts its toxicity, there are disadvantages of these *in vitro* systems for toxicogenomic studies. Previous studies demonstrated that cultured liver cell lines expressed very low or undetectable levels of phase I metabolizing enzymes compared with liver slices or liver (Boess et al., 2003). Hence, cell lines which are used for predicting the toxicity of a compound could lead to misinterpretation of results. Another limitation of applying *in vitro* systems in toxicogenomic studies for the prediction of chronic toxicity is loss of function with long term cultivation of primary cell/tissue culture. In addition, the local microenvironment of the tissue and complex interactions between adjacent tissues are difficult to be modeled in *in vitro* system. Therefore, these *in vitro* systems can not replace whole animal test systems entirely. Although, there are still some caveats and challenges in utilization of toxicogenomics, it is believed that toxicogenomics could offer additional values compared to conventional toxicology methods. Toxicogenomics of zebrafish embryos offer an ethically acceptable methods band on a vertebrate for classification and potential prediction of toxicity of chemicals.

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Appendix

Table 1. Summary of microarray experiments. Embryos were either treated from 4 to 24 or from 24 to 48 or from 96 to 120 hpf. Arrays : total number of microarray hybridization. Number in brackets indicated the number of independent biological repeats.

Toxin	Stage	Concentration			Arrays
4-CA	24 hpf	15 ppm	15 mg/l	118 µM	8 (3)
	48 hpf	50 ppm	50 mg/l	390 µM	6 (3)
	120 hpf	50 ppm	50 mg/l	390 µM	8 (3)
	120 hpf	25 ppm	25 mg/l	195 µM	4 (1)
	120 hpf	5 ppm	5 mg/l	39 µM	4 (1)
	120 hpf	0.5 ppm	0.5 mg/l	3.9 µM	4 (1)
DDT	24 hpf	5 ppm	5 mg/l	14 µM	6 (3)
	48 hpf	15 ppm	15 mg/l	42 µM	6 (2)
	120 hpf	15 ppm	15 mg/l	42 µM	8 (3)
	120 hpf	1.5 ppm	1.5 mg/l	4.2 µM	4 (1)
	120 hpf	0.15 ppm	0.15 mg/l	0.42 µM	4 (1)
Cd	24 hpf	0.5 ppm	0.5 mg/l	2.7 µM	8 (4)
	48 hpf	5 ppm	5 mg/l	27 µM	8 (3)
	120 hpf	5 ppm	5 mg/l	27 µM	8 (3)
	120 hpf	2.5 ppm	2.5 mg/l	13.5 µM	4 (2)
	120 hpf	0.5 ppm	0.5 mg/l	2.7 µM	4 (2)
	120 hpf	50 ppb	50 µg/l	0.27 µM	4 (2)
TCDD	24 hpf	150 ppt	150 ng/l	0.47 nM	8 (3)
	48 hpf	500 ppt	500 ng/l	1.6 nM	4 (2)
	120 hpf	500 ppt	500 ng/l	1.6 nM	8 (3)
	120 hpf	250 ppt	250 ng/l	0.8 nM	4 (1)
	120 hpf	50 ppt	50 ng/l	0.16 nM	4 (2)
VA	24 hpf	15 ppm	15 mg/l	12.9 µM	8 (3)
	48 hpf	50 ppm	50 mg/l	43 µM	8 (3)
	120 hpf	50 ppm	50 mg/l	43 µM	8 (3)
	120 hpf	25 ppm	25 mg/l	21.5 µM	4 (1)
	120 hpf	5 ppm	5 mg/l	4.3 µM	4 (1)
	120 hpf	0.5 ppm	0.5 mg/l	0.43 µM	4 (1)
Hg	24 hpf	50 ppb	50 µg/l	0.20 µM	8 (3)
	48 hpf	60 ppb	60 µg/l	0.24 µM	6 (2)
	120 hpf	60 ppb	60 µg/l	0.24 µM	10 (3)
	120 hpf	30 ppb	30 µg/l	0.12 µM	4 (2)
	120 hpf	6 ppb	6 µg/l	0.024 µM	4 (2)
As	120 hpf	79 ppm	79 mg/l	400 µM	8 (3)
	120 hpf	7.9 ppm	7.9 mg/l	40 µM	4 (1)
Pb	120 hpf	2.8 ppm	2.8 mg/l	10 µM	8 (3)
	120 hpf	0.28 ppm	0.28 mg/l	1 µM	4 (1)
PCB	120 hpf	33 ppm	33 mg/l	100 µM	8 (3)
AA	120 hpf	71 ppm	71 mg/l	1 mM	8 (3)
t-BHQ	120 hpf	1.7 ppm	1.7 mg/l	10 µM	8 (3)
Mixture	120 hpf	Pb,1 µM; Cd,0.27 µM; As,40 µM; Hg,0.024 µM			6 (3)

Table 2. Summary of gene responses of embryo exposed to different concentrations of Cd. Only genes whose expression is twice up or down regulated with Padj≤0.025 were listed.

Name	ID	Cd(5mg/l)	Cd(2.5mg/l)	Cd(0.5mg/l)
GTP cyclohydrolase 1	AJ311846	-3.3	-2.7	-2.6
Thioredoxin	BI864190	4.0	2.9	2.2
unknown	AW019526	-2.5	-2.0	-2.1
unknown	BI887138	2.5	2.5	2.5
Ribosomal protein S17	BI474700	8.6	8.5	4.8
oncomodulin A	BE201681	-4.3	-5.7	-4.5
Solute carrier family 16, member 9	BE016639	2.9	2.4	2.5
Peroxiredoxin 1	BI980610	3.1	2.8	2.3
arginase, type II	BG891983	3.1	2.3	2.1
Alpha-2-HS-glycoprotein	AI496863	-3.3	-2.7	-2.9
sequestosome 1	AW343560	3.9	3.1	2.5
Arginase, type II	BF717769	2.9	2.1	
heat shock cognate 70-kd protein	AF210640	6.6	2.9	
unknown	AI353541	2.8	2.5	
unknown	BG727177	2.2	2.1	
Sulfide quinone reductase-like	BI882244	3.6	3.0	
uncoupling protein 2	AJ243250	2.3	2.5	
Tripartite motif-containing 62	BI878269	2.2	2.4	
Collagen, type VII, alpha 1	AI601501	-2.3	-2.3	
unknown	AW154517		2.1	2.9
Ribonucleotide reductase M2 polypeptide	U57965		-2.9	-2.9
Karyopherin alpha 2	BI878593		-2.4	-2.3
unknown	AI330535		4.5	2.6
Ornithine decarboxylase antizyme 2	BI982208		2.8	2.7
unknown	AW420321		2.2	2.1
Solute carrier family 16, member 9	BI474827		3.5	2.9
unknown	BI886268		-2.2	-2.3
Parvalbumin	BI845755		-3.9	-2.0
unknown	AW115990		2.9	2.4
Protein tyrosine phosphatase type IVA, 3	BM095161		2.4	2.3
hydroxysteroid 11-beta dehydrogenase 2	AW154688		2.8	2.7
phosphoenolpyruvate carboxykinase 1	BG727092		2.2	2.3
Lactate dehydrogenase A	BI983171		2.8	2.3
unknown	AI106316		2.4	2.3
CCAAT/enhancer binding protein (C/EBP), beta	AW019436	2.3		
Tumor necrosis factor, alpha-induced protein 9	BI878750	2.3		
Solute carrier family 43, member 2	AI331043	2.2		
Mannose receptor, C type 2	BI533854	3.8		
Matrix metalloproteinase 9	AW174507	12.3		
unknown	BG728693	2.1		
Lysosomal-associated membrane protein	AI793516	2.6		

2				
membrane protein, palmitoylated 1	AW128294	2.3		
topoisomerase (DNA) II alpha	BM156751	-2.2		
Solute carrier family 6, member 8	BI980828	2.0		
guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2	AY050500	-3.1		
Matrix metalloproteinase 13	AW305943	11.9		
Amylase, alpha 2B	BM103972	-2.2		
Solute carrier family 43, member 2	AW117094	2.0		
unknown	BI878214	3.1		
DnaJ (Hsp40) homolog, subfamily B,4	BM156904	2.5		
unknown	AI353803	-2.4		
unknown	BF717646	-2.1		
Interferon regulatory factor 1	BI326597	2.7		
pyruvate dehydrogenase kinase, isoenzyme 2	BM101544	2.7		
unknown	BI880567	2.0		
unknown	BE605735	2.5		
unknown	AI964296	2.2		
cytochrome P450, family 2	AF283813	3.1		
heat shock cognate 70-kd protein	AB062116	7.0		
unknown	AI641534	3.1		
MAP kinase-interacting serine/threonine kinase 2	BI839784	2.9		
MAP kinase-interacting serine/threonine kinase 2	BI533884	2.8		
Complement component 4B,	BI672168	4.0		
CD44 antigen	AW344023	2.4		
Keratin 13	BE200701	-2.4		
elastase 2	BI705588	-2.4		
guanylate kinase 1	BI670894	-2.1		
hatching enzyme 1	AI877922	-2.0		
Inner centromere protein antigens	BI704423	-2.5		
MCM3 minichromosome maintenance deficient 3	BI889166	-2.1		
unknown	BM096075	2.7		
unknown	AW420509	2.3		
unknown	BI887230	-2.1		
Complement component 7	AA497156	7.4		
MAP kinase-interacting serine/threonine kinase 2	AI657551	2.4		
unknown	BM036361	2.1		
unknown	BM186492	2.1		
unknown	BG727211	2.3		
angiotensinogen	BG727310	2.7		
Immunoglobulin-like domain containing receptor 1	BF157317	2.0		
Suppressor of cytokine signaling 3	BI878700	2.2		
Stathmin 1/oncoprotein 18	BI891936	-2.5		

retinoblastoma binding protein 4	AW116425	-2.1		
Glutathione S-transferase omega 2	BI979918	2.7		
unknown	BE606169	5.0		
Apolipoprotein D	BM101644	-2.0		
Deoxycytidine kinase	AW059347	-2.6		
Claudin 7	AF260240	2.1		
complement component factor B	U34662	2.0		
unknown	AI793733	-2.1		
unknown	AI330865	3.1		
CD99 antigen-like 2	BI888934	-2.1		
unknown	AI330404	3.1		
thioredoxin interacting protein	BI892352	2.0		
unknown	AW280037	2.4		
arginase, type II	AW018735	2.3		
unknown	BI705262	3.3		
Ubiquitin-conjugating enzyme E2T	AW420785	-2.1		
unknown	BI325662	-2.6		
unknown	BG891932	2.1		
unknown	AI477644	3.5		
unknown	AW078445	2.3		
unknown	BI883516	5.5		
unknown	BI892060	2.0		
hypoxia induced gene 1	BI892416	2.7		
Solute carrier family 16, member 9	BI474827	4.3		
Parvalbumin	BI845755	-3.6		
unknown	AW419887	2.1		
unknown	AW280086	2.3		
activating transcription factor 3	AW422298	3.2		
Zinc fingers and homeoboxes 1	AI793802	2.6		
Chemokine (C-X-C motif) ligand 3	BI845861	2.8		
vitellogenin 1	BI841891	2.1		
unknown	BM072227	-2.0		
unknown	AW019018	2.8		
unknown	AI397362	2.5		
unknown	BI896258	-2.4		
Cyclic AMP-regulated phosphoprotein	AI544565	2.4		
Keratin 6	BE016992	-3.1		
unknown	BM184056	2.9		
Suppressor of cytokine signaling 3	BG727181	2.5		
Immunoglobulin superfamily, member 2	BE605911	2.8		
membrane protein, palmitoylated 1	BF158097	2.0		
Syncollin	AA566708	-2.7		
heat shock cognate 70-kd protein	AF006007	6.3		
Ubiquitin-conjugating enzyme E2D 4	BM072375	2.2		
vitellogenin 1	BM036395	2.2		
unknown	BI843139	2.7		
unknown	BI430332	2.1		
apolipoprotein Eb	AJ236882	2.2		

unknown	BI867672	2.5		
unknown	AI354073	2.2		
unknown	BI533153	2.0		
apolipoprotein Eb	BI878442	2.0		
unknown	AI354170	-2.1		
membrane protein, palmitoylated 1	BI839240	2.1		
Arrestin domain containing 3	BF157296		2.1	
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	BI888564		2.0	
cell division cycle 2	BI888928		-2.1	
DNA-damage-inducible transcript 4	BI843145		2.7	
unknown	AI588580		2.1	
4-aminobutyrate aminotransferase	AI882774		2.7	
unknown	AW454405		-2.0	
unknown	AI793855		-2.8	
keratin 4	AF134850		-2.1	
unknown	AL591382		2.1	
unknown	BG727339		2.1	
ataxin 2-binding protein 1-like	AI958253		-2.0	
annexin A1b	AI331515		2.7	
unknown	BM005448		2.5	
unknown	AW116170		-2.2	
proliferating cell nuclear antigen	AF140608			-2.0
unknown	AI397362			2.7
collagen type II, alpha-1	U23822			-2.0
Acyl-Coenzyme A oxidase 1	BM183246			-2.3

Table 3. Summary of gene responses of embryos exposed to different concentrations of MeHg. Only genes whose expression is twice up or down regulated with Padj≤0.025 were listed.

Name or Human HomoloGene	ID	Hg(60µg/l)	Hg(30µg/l)	Hg(6µg/l)
Ribosomal protein S17	BI474700	6.9	4.2	2.2
Peroxiredoxin	BI980610	7.7	4.6	3.5
Glutamate-cysteine ligase	BG304082	2.4	2.1	
Matrix metalloproteinase 13	AW305943	5.9	2.8	
ATP-binding cassette, sub-family C. 2	AW279805	2.4	2.2	
oncomodulin A	BE201681	-2.6	-2.3	
Glutathione S-transferase omega 1	AW019036	2.6	2.7	
Apical early endosomal glycoprotein precursor	BG302634	3.0	2.4	
Glutathione peroxidase 2	AW232474	-4.2	-3.0	
glutathione peroxidase 4a	BI896246	-2.6	-2.8	
glutathione S-transferase pi	AF285098	3.2	2.2	
Suppressor of cytokine signaling 3	BG727181	4.5	2.4	
unknown	AI397362		2.8	2.7

unknown	BM185062	-2.5		
unknown	AW420476	-2.7		
unknown	AI957831	-2.3		
unknown	BI886601	-2.1		
Zinc finger and SCAN domain containing 1	AI667275	-2.2		
unknown	BG306925	-2.3		
Complement component 6	BI474371	3.6		
Tubulin, beta 4	BM186665	-2.8		
unknown	BI979388	-2.4		
Natriuretic peptide precursor A	BE693172	2.1		
unknown	AA542597	-2.0		
Complement component 3	AW116558	2.3		
unknown	BI891316	-2.0		
Thioredoxin	BI864190	4.4		
Prosaposin	AI722545	-3.1		
unknown	AI558658	-3.1		
matrix metalloproteinase 9	AW174507	6.7		
Protein disulfide isomerase-associated	BI885320	-2.1		
unknown	BG728693	2.0		
unknown	AW117109	2.1		
Lysosomal-associated membrane protein 2	AI793516	2.5		
unknown	BI889335	-2.2		
strongly similar to KIAA1143 protein	BI350885	-2.3		
Amylase, alpha 2B; pancreatic	BM103972	-2.1		
unknown	BI878214	2.2		
Cylindromatosis	BE016427	-3.1		
unknown	BI886668	-2.3		
Myeloperoxidase	AF349034	2.5		
unknown	BM182366	-2.2		
Interferon regulatory factor 1	BI326597	2.1		
Cofilin 2 (muscle)	BI883993	3.3		
unknown	AI964296	2.1		
unknown	AW171394	-2.1		
unknown	AI641534	2.0		
Cholinergic receptor, nicotinic, gamma polypeptide	AI793818	2.9		
Complement component 4B	BI672168	5.3		
CD44 antigen	AW344023	2.2		
unknown	AI721719	-2.1		
unknown	BI980805	-2.1		
unknown	AW420546	2.1		
unknown	BI844137	-2.0		
unknown	BM096075	2.2		
Diaphorase (NADH)	AI722339	-2.1		
Complement component 7	AA497156	3.4		
unknown	BM182280	2.4		
unknown	AI641239	2.1		

D component of complement (adipsin)	AW174653	2.0		
unknown	BG727211	3.1		
angiotensinogen	BG727310	3.0		
Suppressor of cytokine signaling 3	BI878700	2.7		
Selenoprotein P, plasma, 1	AF322071	-2.7		
unknown	AW279682	-2.0		
Sirtuin	BI876457	-2.0		
Nuclear RNase III Drosha	BG303805	-2.1		
Deiodinase, iodothyronine, type I	BF717782	-2.9		
unknown	AW115782	2.2		
Solute carrier family 16, member 9	BE016639	2.4		
unknown	BI886819	-2.2		
unknown	BI887093	-2.3		
unknown	BI318394	-2.1		
B-factor, properdin	U34662	3.9		
unknown	AW343746	-2.2		
unknown	BI890893	-2.2		
transketolase	BI865881	-2.1		
Nephronophthisis 1	AW466634	-3.0		
Solute carrier family 16, member 6	AW421040	2.9		
unknown	AI584430	2.0		
Sulfide quinone reductase-like	BI882244	3.1		
Serum/glucocorticoid regulated kinase|	BI673466	2.6		
unknown	BG303804	-2.3		
unknown	BG891932	3.3		
unknown	AI477644	12.4		
unknown	AW058848	-2.1		
Hypoxia up-regulated 1	AW116343	2.2		
unknown	BI886290	2.1		
Proteasome 26S subunit	AI415997	-2.1		
unknown	AW019023	2.3		
unknown	BI981043	2.4		
unknown	BI889194	-2.6		
Zinc finger protein 581	AI723212	-2.2		
unknown	BE558041	2.0		
Prostate cancer antigen-1	BI708772	-2.2		
Leucine zipper transcription factor-like 1	BI879148	-2.0		
Hypoxia up-regulated 1	BI876732	2.3		
TH1-like (Drosophila)	AW174469	-2.2		
Complement component 3	AF047415	2.9		
CCAAT/enhancer binding protein (C/EBP) 1	AF306857	2.6		
unknown	BI878747	-2.2		
hypoxia induced gene 1	BI892416	2.3		
Solute carrier family 16, member 9	BI474827	4.8		
Prostate cancer antigen-1	BI673635	-2.3		
unknown	BI892110	2.7		
unknown	AW128255	-2.6		
unknown	BI887764	-2.0		

RAN binding protein 9	AW059129	-2.1		
transaldolase 1	BI896473	2.2		
Thiopurine S-methyltransferase	BM025250	2.2		
Claudin 23	BI888493	2.0		
Activating transcription factor 3	AW422298	3.3		
Zinc fingers and homeoboxes 1	AI793802	2.7		
unknown	AW115990	3.2		
Chemokine (C-X-C motif) ligand 3	BI845861	3.8		
unknown	BI474299	2.2		
unknown	BG303490	-2.9		
Sequestosome 1	AW343560	3.2		
unknown	AW019018	4.9		
Lectin, galactoside-binding, soluble, 1	BM155827	2.1		
unknown	AI397362	3.2		
Fibronectin 1	BG302581	2.9		
unknown	BI867917	-2.2		
unknown	BM036297	-2.3		
Cyclic AMP-regulated phosphoprotein	AI544565	2.4		
Ribosomal protein L30	BE605410	-2.2		
unknown	BI982778	-2.6		
Immunoglobulin superfamily, member 2	BE605911	2.2		
unknown	AI974163	2.7		
unknown	AI974189	2.1		
Tubulin, beta 4	BI864873	-2.4		
unknown	BI705720	-2.5		
Syncollin	AA566708	-2.3		
ceruloplasmin	AF336125	2.0		
cryptochrome 5	AB042254	2.2		
unknown	BI673353	-2.1		
Mucin 6, gastric	AW077600	2.3		
Thioredoxin-like 4B	BI672089	-2.3		
unknown	BI880385	-2.1		
unknown	AW116284	2.1		
unknown	AA495102	2.2		
complement component c3a	AF047413	2.4		
fibrinogen, gamma polypeptide	BI878442	2.4		
Cofactor of BRCA1	AI959391	-2.2		
unknown	AW019312		2.2	
unknown	BI843539			2.2
Gastric intrinsic factor	AI353694			2.4

Table 4. Summary of gene responses of embryos exposed to different concentrations of TCDD. Only genes whose expression is twice up or down regulated with Padj≤0.025 were listed.

Name or Human HomoloGene	ID	TCDD (500ng/l)	TCDD (250ng/l)	TCDD (50ng/l)
Cytochrome P450 1A1	AF057713	31.6	29.5	5.0
Keratin 15	AI397347	-2.2	-2.4	
Similar to SULT6B1	AI959735	2.8	2.3	
cytochrome P450 1C1	BG738243	12.9	11.3	
unknown	BM183152	3.3	3.0	
unknown	BM101698	2.2	2.3	
unknown	AI397362		2.8	2.7
unknown	AI384221	2.1		
Forkhead box Q1	AW566603	2.1		
Gastric intrinsic factor	AI353694	2.1		
Cytochrome c oxidase subunit IV isoform 2	BI672330	2.3		
unknown	AI353244		-2.1	
Arginase, type II	BF717769		-2.0	
Arrestin domain containing 3	BF157296		-2.1	
unknown	BI326783		-2.2	
unknown	AI958860		-2.1	
Apical early endosomal glycoprotein precursor	BG302634		3.3	
Arginase, type II	AW018735		-2.1	
Arginase, type II	BG891983		-2.2	
unknown	AW019312			2.1

Table 5. Summary of gene responses of embryos exposed to 50 µg/l Cd or 6µg/l MeHg or 7.9mg/l As or 280µg/l Pb alone or to a mixture of them. ID, gene bank accession number.

Name or Human homoloGene	ID	As	Cd	Hg	Pb	Mixture
Peroxiredoxin	BI980610	2.7		3.5	9.6	15.5
Gastric intrinsic factor	AI353694	2.0		2.4	3.3	5.1
kruppel-like factor 2a (klf2a)	AF392992	-2.1	-4.8			-3.9
Period homolog 2 (Drosophila)	BG303941	2.4	2.1		3.5	
Ribosomal protein S17	BI474700		4.3	2.2		10.5
Oncomodulin	BE201681		-4.3		-3.0	-5.5
uncoupling protein 2 (ucp2 gene homologue).	AJ243250		2.1		2.2	4.5
CCAAT/enhancer binding protein (C/EBP), beta	AW019436	-2.3				4.0
Suppressor of cytokine signaling 3	BI878700	-4.9				2.6
Matrix metalloproteinase 13	AW305943		3.2			4.1
Arrestin domain containing 3	BF157296		2.5			2.7
unknown	BI888424		2.1			2.1
Complement component 7	AA497156		4.1			10.1

Stathmin 1/oncoprotein 18	BI891936		-2.2			-3.2
Solute carrier family 16 member 9	BE016639		3.3			3.5
Solute carrier family 16 member 6	AW421040		4.0			4.3
unknown	AW019503		2.2			4.3
gelsolin	AF175294		-2.1			-2.8
DnaJ (Hsp40) homolog, subfamily A, member 1	BI891737		2.3			4.7
unknown	BQ618331		2.5			3.2
unknown	BI325662		-2.1			-3.1
unknown	AL591382		2.5			2.3
Keratin 25D	AI397347		-2.2			-2.4
Solute carrier family 16 member 9	BI474827		2.7			2.8
unknown	BI886268		-2.3			-2.6
Parvalbumin	BI845755		-3.0			-4.1
ornithine decarboxylase	AF290981		2.1			2.9
Activating transcription factor 3	AW422298		2.1			10.2
Alpha-2-HS-glycoprotein	AI496863		-3.2			-6.7
Suppressor of cytokine signaling 3	BG727181		2.2			2.2
thymidylate synthase	AY005804		-2.3			-2.7
Thioredoxin	BI864190				4.7	14.4
unknown	BM182280				2.1	5.5
Glutathione S-transferase omega 2	BI979918				3.7	4.2
unknown	AW115782				2.8	4.5
glutathione S-transferase pi	AF285098				4.3	6.0
Complement component 3	AI588354				2.1	4.0
zinc-finger protein (KROX-24) gene	M81109	-2.3				
btg-b anti-proliferative cofactor	AB036784	-2.2				
unknown	BM102367	-2.2				
Jun dimerization protein 2	BI326453	-4.1				
z64phr 6-4 photolyase	AB042254	2.4				
unknown	BI533153	-2.0				
Similar to Mucin (MUC3)	AI959644		-2.6			
unknown	AW076901		-2.1			
Brachyependymin beta and gamma chains (Epd) gene	M89643		-2.2			
Na+/K+ ATPase alpha1A1 subunit	AY028629		-2.1			
Cofilin 2 (muscle)	BI883993		2.5			
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	BI888564		2.1			
cytochrome P450 monooxygenase CYP2K6	AF283813		2.4			
Dehydrogenase/reductase (SDR family) member 1	AI496835		-2.2			
Alanyl (membrane) aminopeptidase	AI497338		-2.4			
Keratin 13	BE200701		-2.3			
Eukaryotic translation initiation factor 4A, isoform 1	BI876856		-2.3			
FBJ murine osteosarcoma viral oncogene homolog B	BE605888		-2.1			
unknown	AW342722		-2.6			

cytochrome P450 1A1	AF057713		2.1			
unknown	BI896258		-2.7			
Phosphoenolpyruvate carboxykinase 1	BG727092		2.3			
Tripartite motif-containing 62	BI878269		2.1			
unknown	BG304024		2.1			
apoE gene	AJ236882		2.1			
MCM5 minichromosome maintenance deficient 5,	AW058902		-2.0			
unknown	AI106316		2.4			
unknown	BI843539			2.2		
unknown	AI397362			2.7		
ATP-binding cassette, sub-family C member 2	AW279805				2.2	
guanylate cyclase-activating protein 3	AY044457				2.1	
20 beta-hydroxysteroid dehydrogenase	AF298898				2.4	
Glutathione S-transferase omega 1	AW019036				5.0	
Na+/K+ ATPase alpha subunit isoform 3 (atp1a1a.2)	AF286374				-2.2	
heat shock protein hsp90alpha	AF068773					2.6
Similar to 60S ribosomal protein L27a	BI841063					2.1
unknown	BM103939					2.1
brain-type fatty-acid binding protein	AF237712					-2.4
heat shock cognate 70 kDa protein (hsp70) gene	AF006006					2.6
Solute carrier family 2 member 5	AI477656					2.6
unknown	AI626751					2.0
Heme oxygenase (decycling) 1	AI722432					2.8
Tyrosyl-tRNA synthetase	AW777876					2.6
Collagen, type IX, alpha 3	AI601714					-2.5
unknown	AI658234					-3.1
liver-basic fatty acid binding protein	AF254642					-2.5
Solute carrier family 27 (fatty acid transporter), member 2	AI884099					-2.3
unknown	AW117109					3.1
BTG family, member 3	BI979982					2.0
Zinc finger, A20 domain containing 2	BE201929					2.3
Membrane protein, palmitoylated 1, 55kDa	AW128294					2.6
unknown	AW420559					2.2
strongly similar to coiled-coil-helix-coiled-coil-helix domain containing 2	AW117105					2.1
ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	BM186095					4.4
Thioredoxin-like 1	BI886187					2.2
cone transducin alpha subunit	AY050500					-2.4

(gnat2)						
DnaJ (Hsp40) homolog, subfamily B, member 4	BM156904					4.5
Arginase, type II	BF717769					3.2
unknown	BI890954					-2.1
putative cellular retinol-binding protein	AF448140					-2.1
Ankyrin repeat domain 11	AW115851					-2.6
Macrophage migration inhibitory factor	BM183259					-2.2
Optineurin	AW280217					2.4
proliferating cell nuclear antigen (PCNA)	AF140608					-2.6
HSP70 stress protein HSP70	AB062116					12.8
MAP kinase interacting serine/threonine kinase 2	BI839784					3.5
ATP-binding cassette, sub-family A (ABC1), member 1	AW421006					2.7
unknown	BM005308					2.3
MAP kinase interacting serine/threonine kinase 2	BI533884					2.6
Alpha-2-macroglobulin	BI326783					-2.5
green-sensitive opsin 1 (grops1)	AF109369					-2.5
ribonucleotide reductase protein R2 class I	U57965					-3.1
unknown	BM182744					2.6
Collagen, type I, alpha 2	AI331605					-2.1
Hairy and enhancer of split 5	BM072241					-2.0
Heat shock 60kDa protein 1 (chaperonin)	BG985703					2.3
presenilin-2 (ps2)	AF178539					2.6
transcription factor stat3.	AJ005693					2.2
unknown	BI704181					-2.4
Similar to mitochondrial C1-tetrahydrofolate synthase	AW777642					2.4
unknown	AW420509					5.6
Alanine-glyoxylate aminotransferase 2-like 1	AW154396					-2.4
Dihydrodiol dehydrogenase (dimeric)	BI708067					2.4
Brain protein I3|	BM083968					2.0
unknown	BG727310					3.0
Galactosamine (N-acetyl)-6-sulfate sulfatase	BI984280					2.3
Retinoblastoma binding protein 4	AW116425					-2.2
Growth and transformation-dependent protein	BM071848					2.2
unknown	BI878191					2.0
Hsp70 gene	AF210640					8.7
unknown	AI957593					5.3
Fetuin B	BI885905					-4.0
unknown	AL719902					-2.5

Collagen, type IX, alpha 2	AI558471					-2.4
CD63 antigen (melanoma 1 antigen)	AW019276					2.6
unknown	BM024785					2.3
unknown	AI641660					2.4
unknown	AI793855					-2.7
unknown	BM072346					2.7
CD99 antigen-like 2	BI888934					-2.3
Similar to pleckstrin homology domain containing, family M	AI641731					2.2
unknown	BI983434					-2.7
Tribbles homolog 2 (Drosophila)	BI886711					2.5
unknown	AI477982					-2.1
F-box protein 32	BG303968					3.9
unknown	BM182911					-2.0
unknown	AW280037					3.0
Arginase, type II	AW018735					3.5
unknown	BE016756					2.8
BCL2-associated X protein	BI891654					2.0
Keratin 6 irs	AI477659					-2.3
unknown	BI983410					2.4
Follistatin-like 1	AI884233					3.2
Prostaglandin-endoperoxide synthase 2	AW077995					2.6
S100 calcium binding protein A4	AW595487					-2.2
mismatch repair protein Msh6 (msh6)	AF412834					-2.2
Tat-interacting protein Tip30 (tip30)	AF329850					2.6
strongly similar to cardiac muscle alpha actin proprotein	AI601793					-2.0
unknown	AW078445					4.0
Collagen, type XIV, alpha 1 (undulin)	AI584441					-2.7
intestinal fatty acid binding protein (IFABP)	AF180921					-2.9
unknown	BI879661					-2.3
Alpha-2-macroglobulin	BI326782					-2.5
unknown	BI896473					3.1
unknown	AW419887					3.8
unknown	AW280086					2.9
activator of heat shock 90kDa protein ATPase homolog 1	BM103957					2.4
Zinc fingers and homeoboxes 1	AI793802					5.7
unknown	AW115990					7.6
Collagen, type I, alpha 1	BI671608					-2.2
unknown	BM071885					-2.6
MINDIN2	AB006085					-2.1
red-sensitive opsin (rdops)	AF109371					-2.2
Mcl1b (mcl1b)	AF441284					2.2
unknown	BM072227					-2.3

Procollagen-proline, 2-oxoglutarate 4-dioxygenase	AW059030					2.5
Sequestosome 1	AW343560					15.4
unknown	AI331606					-3.0
unknown	BG799399					-2.4
Prestin (motor protein)	BG727321					2.2
unknown	BI882313					-2.2
unknown	BI982778					-2.5
Serine (or cysteine) proteinase inhibitor, clade A	AW018949					-3.1
Syncollin	AA566708					-3.2
dopachrome tautomerase (dct)	AF280090					-2.0
inducible 70 kDa heat shock protein (hsp70) gene	AF006007					11.9
unknown	AI722353					3.3
Alpha-2-macroglobulin	AI957415					-2.0
RasGEF domain family, member 1B	AI723236					2.4
putative delta-6 fatty acyl desaturase (Fadsd6)	AF309556					-3.1
ferritin heavy chain (fth1)	AF295373					2.2
cell death regulator Mcl-1a	AF302805					2.3
unknown	AI878489					2.7
unknown	BI883251					-2.4
unknown	BM035545					2.0
Cardiomyopathy associated 3	BG728392					4.3

Table 6. Summary of gene responses of embryos exposed to 11 different chemicals. Only genes whose expression is twice up or down regulated with Padj≤0.025 were listed.

Gene name	Gene ID	AA	As	CA	Cd	DDT	Hg	Pb	PCB	tBHQ	TCDD	VA
Peroxiredoxin 1	BI980610	3.9	13.6	4.1	3.1		7.7	9.9		7.5		
Thioredoxin	BI864190		14.2	3.5	4.0		4.4	8.1	2.5	6.1		
Stress protein HSP70,	AB062116		10.1	2.2	7.0			2.5	5.4	12.3		2.7
Activating transcription factor 3	AW422298		4.0		3.2		3.3	2.5	15.1	5.1		5.4
Zinc fingers and homeoboxes 1	AI793802		2.5		2.6		2.7	2.4	4.2	2.1		2.0
Sequestosome 1	AW343560		10.3	2.1	3.9		3.2	3.5	2.8	5.1		
unknown	BG985532	2.6	8.8	2.3				4.3	2.6	2.9		
Glutathione S-transferase omega 1	AW019036	3.0	6.3	2.1			2.6	3.5		2.2		
matrix metalloproteinase 9	AW174507		2.1		12.3		6.7	3.9	14.2	6.4		
Hsp70	AF210640		10.9		6.6			2.5	5.0	10.8		2.7
6-Phosphogluconate dehydrogenase	AW115782		3.3	2.0			2.2	3.4	2.0	2.1		
Likely ortholog of mouse hypoxia induced gene 1	BI892416		2.6		2.7		2.3	3.4	2.7	2.2		
Fetuin-A,	AI496863		-3.6	-2.2	-3.3			-3.2	-5.0			-2.3

unknown	AW115990		3.1				3.2	2.3	5.6	4.6		3.1
Hsp70	AF006007		7.6		6.3			2.1	4.6	10.1		2.4
Matrix metalloproteinase 13 (collagenase 3)	AW305943			2.5	11.9		5.9	3.5	15.1	10.2		
Angiotensinogen precursor,	BG727310			3.0	2.7		3.0	2.1	3.7	2.1		
Solute carrier family 16 member 9	BE016639			2.5	2.9		2.4	3.6	4.1			2.3
Solute carrier family 16 member 9	BI474827			3.4	4.3		4.8	4.7	7.9			3.0
Glutathione S-transferase pi	AF285098	2.0	4.1				3.2	5.9		2.7		
CCAAT/enhancer binding protein (C/EBP), beta	AW019436		2.1		2.3			2.4	6.9	2.3		
unknown	AW117109		2.3				2.1	2.1	2.3	3.2		
unknown	AI964296		2.1		2.2		2.1	2.1	2.0			
Syncollin	AA566708		-2.1		-2.7		-2.3	-3.8				-2.2
Cofilin 2 (muscle)	BI883993			2.4			3.3	3.6	3.1			2.2
unknown	BG727211			3.4	2.3		3.1		2.4	2.7		
Serum/glucocorticoid regulated kinase 2	BI673466			2.0			2.6	2.2	3.6			2.0
Cytochrome P450, family 1, subfamily A, polypeptide 1	AF057713			3.7		2.5			4.2	2.8	31.6	
unknown	AW019018			3.6	2.8		4.9		2.5	5.9		
Suppressor of cytokine signaling 3	BG727181			2.0	2.5		4.5		5.6	3.7		
Complement component 7	AA497156				7.4		3.4	6.2	7.3	3.7		
unknown	AI477644				3.5	-2.2	12.4		46.3			8.0
Gastric intrinsic factor (vitamin B synthesis)	AI353694	2.2	4.6					2.5			2.1	
Multidrug resistance-associated protein Mrp2,	AW279805		2.6	2.4			2.4	2.3				
Arsenite inducible RNA associated protein,	BM182280		5.1				2.4	2.5		2.7		
Glutathione S-transferase omega 2	BI979918		5.6		2.7			5.2		2.1		
Pvalb3a	BE201681		-2.4		-4.3		-2.6	-5.1				
unknown	AI330865		2.2		3.1				3.1	3.6		
unknown	BI896473		2.2				2.2	2.5		2.0		
Arginase, type II	BF717769			2.2	2.9			2.2	4.1			
Interferon regulatory factor 1	BI326597			2.1	2.7		2.1					2.3
Complement factor B precursor	U34662			2.7	2.0		3.9			4.6		
Arginase, type II	BG891983			2.6	3.1			2.5	4.9			
similar to UP|Q86V31 (Q86V31) ARPP-21 protein	AI544565			2.5	2.4		2.4		2.3			
Opsin 1 (cone pigments), short-wave-sensitive	AF109373			-2.0					-2.6	-2.7		-2.7
GTP cyclohydrolase 1 (dopa-responsive dystonia)	AJ311846				-3.3			-2.3	-2.2	-2.1		
Guanine nucleotide binding protein alpha transducing activity polypeptide 2	AY050500				-3.1			-2.2		-3.6		-2.7
unknown	BM096075				2.7		2.2		4.3	2.6		
similar to human ribosomal protein S17	BI474700				8.6		6.9		4.3			4.7
Suppressor of cytokine signaling 3	BI878700				2.2		2.7		3.4	3.1		
unknown	BG891932				2.1		3.3		3.0	2.7		

unknown	BM184056				2.9			2.1	3.2	2.2		
unknown	BE605911				2.8		2.2	2.8	2.1			
Natriuretic peptide precursor A	BE693172						2.1	2.1	2.1			2.2
Solute carrier family 16 member 6	AW421040						2.9	4.0	4.6			2.9
Solute carrier family 2 member 5	AI477656		2.1					2.1	2.5			
GTP binding protein 1	AW154231		2.9						2.7			2.1
DnaJ (Hsp40) homolog, subfamily B, member 1,	BM156904		3.5		2.5					4.1		
unknown	AW420509		4.5		2.3			3.3				
30S ribosomal protein S11,	BM101516		2.6						4.3			2.4
Thiopurine S-methyltransferase	BM025250		3.5				2.2	2.6				
V-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	BI887732		2.3					2.4	2.3			
Delta-5/delta-6 fatty acid desaturase	AF309556		-2.3						-3.5			2.1
Cardiomyopathy associated 3	BG728392		4.6						9.9	2.3		
unknown	AW280037			2.1	2.4				6.1			
Arginase, type II	AW018735			2.8	2.3				4.1			
Interferon regulatory factor 7	BE605965			2.3					7.9			6.8
Red-sensitive opsin	AF109371			-2.2					-2.2	-3.3		
unknown	AI974163			2.7			2.7			2.7		
unknown	BG728693				2.1		2.0		2.3			
Lysosomal-associated membrane protein 2	AI793516				2.6		2.5		2.0			
Membrane protein, palmitoylated 1	AW128294				2.3			2.1	2.2			
Solute carrier family 6 member 8	BI980828				2.0			2.2	2.4			
Amylase, alpha 2B; pancreatic	BM103972				-2.2		-2.1	-2.1				
CPG DNA METHYLASE	AI641534				3.1		2.0			3.2		
Complement component 4B	BI672168				4.0		5.3			6.5		
Sulfide quinone reductase-like (yeast)	BI882244				3.6		3.1					2.3
Uncoupling protein 2	AJ243250				2.3			3.4	2.7			
Parvalbumin isoform 2a	BI845755				-3.6			-3.2				-2.6
unknown	AI397362				2.5		3.2			2.8		
Keratin 6	BE016992				-3.1			-2.0		-2.5		
Fibrinogen, gamma polypeptide	BI878442				2.0		2.4			2.1		
Collagen, type VII, alpha 1	AI601501				-2.3				-2.7	-2.0		
Complement component 6	BI474371						3.6		2.2	2.2		
unknown	BI981043						2.4		2.4	2.0		
unknown	BE558041						2.0		3.1	2.5		
F-box protein 32	BG303968							2.4	8.3			2.8
Ornithine decarboxylase antizyme 2	BI982208							2.7	3.1			2.2
Keratin 25D	AI397347							-2.5			-2.2	-2.8
unknown	BI888548	2.5							3.4			
Alanyl (membrane) aminopeptidase	AI497338	2.0		2.1								

ATP synthase, H+ transporting, delta subunit	BM186095		3.0					2.4				
unknown	AI958860		2.3						2.3			
similar to Cyp2a4 protein [	AW421020		-2.2						-2.4			
unknown	AI641239		2.0				2.1					
Selenoprotein P, plasma, 1	AF322071		-2.1				-2.7					
Carbonyl reductase 1	AF298898		2.2					2.1				
Fetuin B	BI885905		-2.1						-2.9			
DnaJ (Hsp40) homolog, subfamily A, member 1	BI891737		3.6							3.6		
unknown	BG302934		-2.0						-2.5			
unknown	BQ618331		2.4					2.5				
Tat-interacting protein Tip30,	AF329850		2.8					2.2				
similar to NADH dehydrogenase subunit 4	AW078445		2.9		2.3							
unknown	AI965077		3.1					2.8				
Ahsa1l protein	BM103957		2.2							2.1		
unknown	BI879928		2.1						2.4			
Eph-like receptor tyrosine kinase rtk5	AJ005026		2.9					4.2				
Complement component 3	AI588354		4.9					2.9				
Beta-2-microglobulin	L05383		3.1					3.8				
unknown	AI878489		2.9						2.9			
Rh50-like protein,	AF209468		-2.3						-2.2			
unknown	AW826221			2.2					2.2			
unknown	BE017917			-2.5					-2.0			
Ribonucleotide reductase M2 polypeptide	U57965			-2.2					-2.7			
unknown	BM036361			2.1	2.1							
unknown	BG883326			2.1								2.6
unknown	AW421018			2.5					7.8			
Cytochrome P450, family 1, subfamily A, polypeptide 1	BG738243			3.1							12.9	
unknown	AW232171			2.1					4.1			
unknown	BI867396			2.2					3.3			
Phosphoenolpyruvate carboxykinase 1 (soluble)	BG727092			2.4					3.7			
Mannose receptor, C type 2	BI533854				3.8					2.9		
similar to UP|Q13641 (Q13641) 5T4 oncofoetal antigen precursor	AW117094				2.0				2.5			
unknown	BI878214				3.1		2.2					
Pyruvate dehydrogenase kinase, isoenzyme 2	BM101544				2.7				2.1			
unknown	BI887138				2.5			2.4				
unknown	BI880567				2.0				2.0			
MAP kinase interacting serine/threonine kinase 2	BI839784				2.9			2.0				
MAP kinase interacting serine/threonine kinase 2	BI533884				2.8				2.3			
unknown	AW344023				2.4		2.2					
Keratin 13	BE200701				-2.4			-2.2				
Ela2 protein	BI705588				-2.4			-3.8				
unknown	BI889166				-2.1				-2.9			

DNA replication complex GINS protein PSF2,	BI887230				-2.1				-2.7			
Retinitis pigmentosa 9 protein	AI657551				2.4				2.8			
Stathmin 1/oncoprotein 18	BI891936				-2.5				-2.8			
Apolipoprotein D	BM101644				-2.0			-2.1				
Deoxycytidine kinase	AW059347				-2.6				-2.8			
unknown	AI793733				-2.1				-2.1			
Thioredoxin interacting protein	BI892352				2.0							2.0
unknown	BI705262				3.3	2.2						
unknown	BI325662				-2.6				-2.3			
unknown	AW419887				2.1			2.3				
unknown	AW280086				2.3				7.8			
Chemokine (C-X-C motif) ligand 3	BI845861				2.8		3.8					
Vitellogenin 1	BI841891				2.1							2.1
Ubiquitin-conjugating enzyme E2D 4	BM072375				2.2				2.4			
unknown	BM036395				2.2				2.3			
unknown	BI867672				2.5							2.5
unknown	BI533153				2.0				3.6			
unknown	AI354170				-2.1			-2.0				
unknown	BI839240				2.1				2.1			
Glutamate-cysteine ligase, modifier subunit	BG304082						2.4		2.4			
Tubulin, beta 4	BM186665						-2.8					-2.0
Complement component 3	AW116558						2.3			2.8		
Myeloperoxidase	AF349034						2.5			2.2		
unknown	AW019023						2.3	2.7				
Complement component 3	AF047415						2.9			3.1		
unknown	BI474299						2.2					2.0
unknown	BE605410						-2.2					-2.0
complement component C3A	AF047413						2.4			3.0		
unknown	AW154517							2.2	3.9			
Kruppel-like factor 2 (lung)	AF392992							-2.3	-2.5			
unknown	BF157296							2.5	2.1			
Ornithine decarboxylase 1	AF290981							3.0	3.4			
unknown	BI839678							-2.1	-2.3			
Chymotrypsin C (Caldecrin)	AW174168							-3.2				-2.1
unknown	BI670896								2.8			2.5
H1 histone family, member X	AW280290								2.7			2.1
Green sensitive cone opsin	AF109369								-2.0	-2.4		
Gelsolin,	AF175294								-2.1	-2.5		
Stathmin-like 4	BG308624								2.2			3.0
SPANX-N2 protein	BI889255								3.5			2.4
unknown	BM183152								-2.4		3.3	
unknown	BI706384								-2.6	-6.2		
Tissue inhibitor of metalloproteinase 3	BM023829								2.5			2.0
Guanine nucleotide binding protein (G protein), beta	BI879650									-2.1		-2.6

polypeptide 3												
unknown	BM101640	-2.2										
unknown	BG883709	2.1										
Alkyl-dihydroxyacetonephosphate synthase precursor	BI709370	-2.9										
Heat shock protein HSP 90-alpha	AF068773		2.6									
unknown	AI497477		-2.1									
unknown	AF006006		2.5									
unknown	AI658234		-2.5									
Monoacylglycerol O-acyltransferase 1	BI892323		-2.3									
unknown	AI641383		2.1									
unknown	AI959120		-2.6									
unknown	AW019145		-2.3									
Apolipoprotein A-IV	AI477980		-2.0									
unknown	BG303673		-2.1									
Alanine-glyoxylate aminotransferase 2-like 1	AW154396		-2.0									
Dihydrodiol dehydrogenase (dimeric)	BI708067		2.1									
unknown	BG883651		-2.1									
unknown	BM072262		-2.1									
Heat shock cognate 70 kDa protein	BM024785		3.0									
Keratin 6	AI477659		-2.1									
unknown	BF938011		2.8									
unknown	AW420765		2.1									
unknown	AW232145		2.1									
Fatty acid binding protein 2, intestinal	AF180921		-2.4									
Membrane-bound aminopeptidase P	BG304004		-2.0									
unknown	AI957765		2.5									
unknown	AW202621		2.1									
unknown	BG799351		-2.1									
unknown	BI880804		2.2									
Proteasome (prosome, macropain) subunit, beta type, 8	AF155578		2.1									
unknown	AW184521		-2.0									
unknown	AW019312		-2.2									
DNA-damage-inducible transcript 4	BI843145			-2.8								
Apolipoprotein B (including Ag(x) antigen)	AI943057			-2.2								
Microsomal NAD+-dependent retinol dehydrogenase 4	AW019006			2.2								
Tumor necrosis factor, alpha-induced protein 9	BI878750				2.3							
unknown	AI331043				2.2							
Topoisomerase (DNA) II alpha	BM156751				-2.2							
unknown	AW019526				-2.5							
unknown	AI353803				-2.4							

unknown	BF717646				-2.1							
unknown	BE605735				2.5							
Cytochrome P450 monooxygenase CYP2K6	AF283813				3.1							
Guanylate kinase 1	BI670894				-2.1							
High choriolytic enzyme,	AI877922				-2.0							
unknown	BI704423				-2.5							
unknown	BM186492				2.1							
unknown	BF157317				2.0							
Retinoblastoma binding protein 4	AW116425				-2.1							
unknown	BE606169				5.0							
Claudin 7	AF260240				2.1							
unknown	AI353541				2.8							
unknown	BG727177				2.2							
Mic2l1	BI888934				-2.1							
unknown	AI330404				3.1							
Ubiquitin-conjugating enzyme E2T (putative)	AW420785				-2.1							
similar to Serum amyloid A protein	BI883516				5.5							
unknown	BI892060				2.0							
unknown	BM072227				-2.0							
unknown	BI896258				-2.4							
unknown	BI878269				2.2							
unknown	BF158097				2.0							
unknown	BI843139				2.7							
unknown	BI430332				2.1							
Apolipoprotein E precursor (Apo-E)	AJ236882				2.2							
unknown	AI354073				2.2							
unknown	AI385015					4.0						
unknown	BM103895					2.2						
unknown	AI384392					3.1						
unknown	AF272963					2.1						
unknown	BM070515					2.7						
Odorant receptor 9.1	AF283560					2.7						
Dynamo protein precursor,	X99769					2.3						
unknown	BI866476					4.6						
unknown	BM185062						-2.5					
unknown	AW420476						-2.7					
unknown	AI957831						-2.3					
unknown	BI886601						-2.1					
unknown	AI667275						-2.2					
unknown	BG306925						-2.3					
unknown	BI979388						-2.4					
unknown	AA542597						-2.0					
unknown	BI891316						-2.0					
unknown	AI722545						-3.1					
unknown	AI558658						-3.1					
unknown	BI885320						-2.1					

unknown	BI889335						-2.2					
unknown	BI350885						-2.3					
unknown	BE016427						-3.1					
unknown	BI886668						-2.3					
unknown	BM182366						-2.2					
unknown	AW171394						-2.1					
unknown	AI793818						2.9					
unknown	AI721719						-2.1					
unknown	BI980805						-2.1					
unknown	AW420546						2.1					
unknown	BI844137						-2.0					
NADH-cytochrome b5 reductase	AI722339						-2.1					
Serine protease-like protein precursor,	AW174653						2.0					
unknown	AW279682						-2.0					
Sirtuin 2 (Silent mating type information regulation 2, homolog) 2,	BI876457						-2.0					
unknown	BG303805						-2.1					
iodothyronine deiodinase type I	BF717782						-2.9					
unknown	BI886819						-2.2					
unknown	BI887093						-2.3					
unknown	BI318394						-2.1					
unknown	AW343746						-2.2					
unknown	BI890893						-2.2					
unknown	BI865881						-2.1					
unknown	AW466634						-3.0					
unknown	AI584430						2.0					
unknown	BG302634						3.0					
unknown	BG303804						-2.3					
unknown	AW058848						-2.1					
Hypoxia up-regulated 1	AW116343						2.2					
Taldo1 protein, c	BI886290						2.1					
unknown	AI415997						-2.1					
unknown	BI889194						-2.6					
unknown	AI723212						-2.2					
glutathione peroxidase	AW232474						-4.2					
Prostate cancer antigen-1	BI708772						-2.2					
Leucine zipper transcription factor-like 1	BI879148						-2.0					
Hypoxia up-regulated 1	BI876732						2.3					
unknown	AW174469						-2.2					
Myeloid-restricted CCAAT/enhancer-binding protein 1,	AF306857						2.6					
unknown	BI878747						-2.2					
Prostate cancer antigen-1	BI673635						-2.3					
unknown	BI892110						2.7					
unknown	AW128255						-2.6					
unknown	BI887764						-2.0					

unknown	AW059129						-2.1					
phospholipid hydroperoxide glutathione peroxidase A	BI896246						-2.6					
unknown	BI888493						2.0					
unknown	BG303490						-2.9					
Proto galectin Gal1-L2,	BM155827						2.1					
unknown	BG302581						2.9					
unknown	BI867917						-2.2					
unknown	BM036297						-2.3					
unknown	BI982778						-2.6					
unknown	AI974189						2.1					
Tubulin, beta, 2,	BI864873						-2.4					
unknown	BI705720						-2.5					
Ceruloplasmin,	AF336125						2.0					
Cry5 protein	AB042254						2.2					
unknown	BI673353						-2.1					
unknown	AW077600						2.3					
unknown	BI672089						-2.3					
unknown	BI880385						-2.1					
Chaperone protein GP96	AW116284						2.1					
unknown	AA495102						2.2					
Cofactor of BRCA1	AI959391						-2.2					
ZEFEPEN ependymin	M89643							-3.2				
unknown	BI868150							2.2				
UDP glycosyltransferase 2 family, polypeptide B7	AW127886							2.2				
unknown	AI384206							-2.1				
unknown	AI958489							-3.8				
unknown	AI793508							-2.1				
Myelin protein zero	AI667023							-3.0				
unknown	BM036460							2.0				
carboxyl ester lipase	AF003943							-2.0				
unknown	BM005137							-2.2				
ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide	AF286374							-3.8				
Similar to peptidyl-Pro cis trans isomerase	AW567464							-2.2				
unknown	BM186973								-2.1			
unknown	AW154454								-2.2			
Cyclin-selective ubiquitin carrier protein E2-C	BE558133								-2.1			
unknown	BM025101								2.1			
unknown	AI942960								2.0			
unknown	AF237712								-2.6			
unknown	AI585113								-3.9			
unknown	BI882477								2.5			
unknown	BM154668								2.0			
unknown	AI522421								2.1			
unknown	BM104369								-2.2			
unknown	AW018635								-2.1			

unknown	BI888241								2.6			
unknown	BI886200								-2.3			
Fatty acid binding protein 6, ileal (gastrotropin)	AF254642								-2.9			
unknown	BI882450								2.5			
unknown	BI671128								2.1			
Thymidine kinase 1	AW116726								-2.1			
DNA methyltransferase 3	BI886329								-2.2			
Ecrg4-A protein,	BI844226								2.0			
unknown	BM095379								-2.5			
unknown	BI843105								-2.2			
unknown	AW058891								2.1			
unknown	AI974197								3.4			
Solute carrier family 15, member 4	BI866561								2.2			
Ankyrin repeat domain 11	AW115851								-2.0			
unknown	BI866278								2.3			
unknown	AW280217								2.4			
unknown	BI888424								2.5			
glutathione peroxidase	AW232570								3.1			
unknown	AI878420								2.2			
Proliferating cell nuclear antigen (PCNA	AF140608								-2.9			
Protein tyrosine phosphatase, receptor type, N	AF190144								2.0			
MKK3 (Mitogen-activated protein kinase kinase 3)	AB030899								-2.3			
Protein kinase, cAMP-dependent, regulatory, type I, alpha	BE557009								-2.5			
Alpha-2-macroglobulin	BI326783								-2.4			
unknown	BI705537								-2.5			
Cell division control protein 2	BI888928								-2.2			
unknown	BM182744								3.4			
F-box protein 2	BI879965								-2.0			
unknown	BI878593								-2.8			
Activating transcription factor 5	AW420576								2.1			
Ephx1 protein	BI704181								-2.1			
Heterogeneous nuclear ribonucleoprotein L	BI889298								-2.0			
unknown	BI983342								-2.1			
flap structure-specific endonuclease 1	AI957820								-2.0			
unknown	BI475933								2.6			
Blue-sensitive opsin	AF109372								-2.6			
unknown	AI626605								2.0			
Period homolog 2 (Drosophila)	BG303941								3.5			
unknown	AI883944								2.1			
unknown	AI584429								-2.2			
unknown	BG308412								2.4			
unknown	BE016173								2.2			

Beta ig-h3	BF717383								-2.2			
Connective tissue growth factor precursor	BE693178								2.5			
unknown	BM154097								2.5			
unknown	AL719902								-2.1			
unknown	BM070610								2.1			
unknown	AI588560								-2.2			
unknown	BI982101								2.6			
unknown	AI877518								-3.4			
Gonadotropin releasing hormone precursor,	AJ304429								2.0			
unknown	BI880600								2.1			
Ribonucleoside-diphosphate reductase M1 chain	U57964								-2.3			
unknown	AI641731								2.2			
unknown	BI983434								-2.4			
Tribbles homolog 2 (Drosophila)	BI886711								3.6			
unknown	AI878005								-2.5			
Response gene to complement 32	BI888368								2.9			
unknown	BI880133								2.3			
unknown	BI883408								2.2			
Succinate-CoA ligase, GDP-forming, beta subunit	BF717706								-2.2			
unknown	AI354010								-2.1			
unknown	BE015932								3.1			
F-box protein 8	BG799020								2.1			
unknown	BG884516								2.4			
unknown	BI983410								2.1			
follistatin-like 1	AI884233								2.4			
Prostaglandin G/H synthase 2	AW077995								3.2			
unknown	AI793707								2.2			
unknown	AI544644								2.6			
MutS homolog 6	AF412834								-2.2			
unknown	AL591382								3.9			
unknown	AI584441								-2.1			
Solute carrier family 43, member 2	BI887324								2.1			
Ring finger protein 38	AI666928								2.2			
unknown	BG727383								2.4			
Solute carrier family 3	BG985518								2.1			
Component of oligomeric golgi complex 2	BG728947								2.3			
Alpha tubulin	AI877819								-2.0			
unknown	BI326782								-2.2			
unknown	AW342722								-2.1			
unknown	BI886268								-2.5			
unknown	BM082387								2.0			
unknown	BE016163								4.4			
MMMNK1 map kinase interacting kinase	BI705604								3.3			

unknown	AW019245								2.1			
unknown	BG727339								2.2			
unknown	BG883210								-2.8			
unknown	AI721732								2.1			
Sprouty homolog 4 (Drosophila)	AF371368								2.7			
Type II Na/Pi cotransport system protein,	AF121796								-2.7			
Myeloid cell leukemia sequence 1	AF441284								2.2			
RNA-binding protein	AI958253								-2.2			
Ribonucleotide reductase M2 polypeptide	AI957409								-3.4			
unknown	BM071693								2.8			
Jun dimerization protein 2	BI326453								3.5			
unknown	AI331606								-2.1			
Protein tyrosine phosphatase type IVA, member 3	BM095161								2.3			
unknown	BG303759								2.1			
unknown	BI865702								2.1			
unknown	BG985441								-2.2			
Cyclin B2	AB040435								-2.4			
Brain-derived neurotrophic factor|	U42489								3.0			
unknown	BG728644								2.2			
MAM domain containing 2	BI866865								3.4			
unknown	BI672160								2.0			
Prestin (motor protein)	BG727321								2.0			
Cytochrome b5	AI558444								2.1			
APEX nuclease (multifunctional DNA repair enzyme) 1	BI883953								-2.3			
Pim-3 oncogene	AF062643								3.9			
unknown	AI722353								7.3			
unknown	BI983171								2.3			
unknown	AI723236								2.7			
unknown	BM005448								2.1			
Cytochrome P450	AF248042								-2.6			
Thymidylate synthetase	AY005804								-3.3			
Cell death regulator Mcl-1a,	AF302805								3.0			
Solute carrier family 20 (phosphate transporter), member 1	BI890772								2.2			
MCM5 minichromosome maintenance deficient 5	AW058902								-2.8			
unknown	BI883251								-2.6			
unknown	AI105923								2.3			
Replication protein A2,	AY007304								-2.5			
Myogenin	AF202639								2.2			
Nuclear transcription factor Y, beta	BM070524								2.5			
Serine/threonine-protein kinase PLK1	AW116681								-2.1			
unknown	BM777961								2.1			

Deoxycytidine kinase	AW203020								-2.3			
Creatine kinase mitochondrial isoform precursor,	AW116371									-2.6		
Retina and anterior neural fold homeobox	AF001908									-2.3		
unknown	BI879707									-2.6		
Tyrosinase ,	AJ250302									-2.2		
Tyrosinase-related protein 1	BI882649									-3.9		
Retinol binding protein 3, interstitial	X85957									-2.1		
Retinol binding protein 1, cellular	AW018731									-2.0		
unknown	BE016208									-3.0		
Complement component 9	AW019201									2.3		
unknown	BI670844									-2.2		
unknown	BG305908									-2.5		
Retinal pigment epithelium-specific protein 65kDa	BI670861									-4.2		
Silver homolog (mouse)	AW076688									-3.7		
Silver homolog (mouse)	AW232707									-3.1		
Retinal G protein coupled receptor	AI497566									-2.5		
unknown	BI883231									-2.3		
Photoreceptor protein s26	BI670920									-2.1		
Dopachrome tautomerase	AF280090									-2.4		
unknown	BG305033									-4.0		
Gngt2 protein	AY050507									-2.4		
unknown	AI384221										2.1	
Forkhead box Q1	AW566603										2.1	
Sulfotransferase	AI959735										2.8	
unknown	BM101698										2.2	
Cytochrome c oxidase subunit IV isoform 2	BI672330										2.3	
unknown	AW280011											2.1
Stathmin-like 4	BI430135											2.1
unknown	BI888755											-2.1
unknown	AI959644											-2.2
Mitogen-activated protein kinase phosphatase x	BI888527											2.3
unknown	BI474304											2.1
unknown	BI702683											2.2
Lipocalin-type prostaglandin D synthase-like protein,	BE017457											2.4
unknown	BM035924											2.1
unknown	BI886755											2.2
Solute carrier family 6 (GABA transporter), member 1	BF157011											-3.1
Cathepsin D	BG303005											2.1
Protein kinase (cAMP-dependent, catalytic) inhibitor alpha	BI863877											2.6
Angiopoietin-like 3	AW421268											2.0

unknown	AI793607											2.2
unknown	AW777561											2.4
Kruppel-like factor 10	AI641738											2.2
LIM and cysteine-rich domains protein 1,	BG799149											2.1
unknown	BM037052											2.2
unknown	BG728726											2.0
unknown	AI545224											2.1
Solute carrier family 6 (transporter, GABA), member 1	BI563084											-2.1
unknown	BI866300											2.2
unknown	BI889456											-2.3
unknown	BI865754											-2.0
unknown	AL590147											2.2
unknown	AW280656											-2.0
unknown	BM156045											2.2
ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide	AF308599											-2.3
unknown	BI673984											2.3
Gamma crystallin M3	BM154967											2.8
unknown	AI617260											2.7
unknown	BG308220											-2.0
unknown	BI896367											2.3
Nr0b2 protein	BI892036											-2.0
Neuroglobin,	AJ315610											-2.2
Histone 3, H2a	AI667325											-2.0
unknown	BI533455											-2.0
unknown	BM141538											2.0