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Chemical-sensitive genes in zebrafish (*Danio rerio*) early development - identification and characterisation of differential expression in embryos exposed to the model compound 3,4-dichloroaniline

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CHEMICAL-SENSITIVE GENES IN ZEBRAFISH (*DANIO RERIO*) EARLY DEVELOPMENT – IDENTIFICATION AND CHARACTERISATION OF DIFFERENTIAL EXPRESSION IN EMBRYOS EXPOSED TO THE MODEL COMPOUND 3,4-DICHLOROANILINE

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„Entwicklung eines Fischembryotests als Alternative für verlängerte und chronische Fischtests: Analyse toxischer Wirkungen auf der Basis veränderter Genexpression im *Danio rerio*-Embryotest (Gen-*Dar*T)“

„Teilprojekt 1: Sensitive Markergene“

durchgeführt. Gen-*Dar*T wurde durch das Bundesministerium für Bildung und Forschung (BMBF) im Rahmen der Förderrichtlinie „Ersatzmethoden zum Tierversuch“, im Programm der Bundesregierung „Biotechnologie – Chancen nutzen und gestalten“ (FKZ 0313016) gefördert.

Die Weisheit der Schöpfung
erkennt man daran,
dass die Fische stumm sind.
Was gäbe es sonst
für einen Lärm,
wenn sie über jedes Ei
gackern würden.

Fritz Kortner (1892-1970)

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ABBREVIATIONS

AHR2	aryl hydrocarbon receptor 2
alpha-2-mg	alpha-2-macroglobulin
ANOVA	analysis of variance
ARE	aryl hydrocarbon receptor response element
aRNA	antisense RNA
ARNT	aryl hydrocarbon receptor nuclear translocator
bp	base pairs
BNF	β-naphthoflavone
c7-1	proteasome component c7-1
cDNA	complementary deoxyribonucleic acid
CT	threshold cycle
CYP1A	cytochrome P450 1A
d	SAM score
*Dar*T	*Danio rerio* embryo toxicity test
3,4-DCA	3,4-dichloroaniline
DMSO	dimethylsulfoxide
dpf	days post fertilisation
DTT	dithiothreitol
E	PCR amplification efficiency
EC	effect concentration
EC_{50}	concentration, exhibiting an effect of 50 % of all organisms
ELST	early life stage test
EpRE	electrophile response element
EST	expressed sequence tag
FDR	false discovery rate
FITC	fluorescein isothiocyanate
FZR1	fizzy related protein 1
Gene-*Dar*T	gene expression *Danio rerio* embryo toxicity test
GFP	green fluorescence protein
HO-1	heme oxygenase 1
hpf	hours post fertilisation
LB	Luria Bertani
LC_{50}	concentration, exhibiting a lethality of 50 % of all organisms

LOEC	lowest observed effect concentration
LOWESS	locally weighted least squares
MAFt	v-maf musculoaponeurotic fibrosarcoma oncogene t
MO	morpholino (antisense oligonucleotides)
MAPK	mitogen-activated protein kinase
mm-siRNA	mismatch short interfering RNA
mRNA	messenger ribonucleic acid
MS 222	tricaine methane sulfonate, ethyl 3-aminobenzoate methanesulfonic acid salt
NOEC	no observed effect concentration
RNS	reactive nitrogen species
NRF2	nuclear factor E2 p45-related factor 2
nt	nucleotide
oligo-dT	oligo desoxythymidine
PAH	polycyclic aromatic hydrocarbon
hPAH	halogenated polycyclic aromatic hydrocarbon
qPCR	quantitative real-time reverse transcriptase polymerase chain reaction
RE	regulatory element
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SAM	significance analysis of microarrays
siRNA	short interfering RNA
TCDD	2,3,7,8-tetrachloro-[p]-dibenzodioxin
U	units
XME	xenobiotic metabolizing enzyme
XRE	xenobiotic response element

SUMMARY

In the European Union an environmental risk assessment is required for the registration of new chemicals, biocides, pesticides and pharmaceuticals. In order to avoid the release of potential hazardous substances, various ecotoxicity tests are performed, including acute and chronic fish tests. As a consequence of the new program of the European Union "Registration, Evaluation and Authorisation of Chemicals" (REACH) the number of animal experiments for environmental risk assessment is expected to increase remarkably within the next years. On the other hand there is a strong societal demand for reducing the number of animal tests by using alternative *in vitro* models.

According to EU directives, investigations using non-human vertebrate embryos are considered pain free *in vitro* methods and are therefore accepted as alternatives to animal experiments. For the acute fish test, the *Danio rerio* embryo test (*Dar*T) has been established as a replacement method and included in national regulations at least for waste water (German Waste Water Dues Law). However, no alternatives for chronic fish tests are currently available. The overall goal of this thesis was to work towards such a replacement by extending *Dar*T zu Gene-*Dar*T. Toxicants will initially interact at the molecular level with consequences for physiology, fitness and survival. The analysis of gene expression patterns may unravel elements of these molecular events before any phenotypic changes are visible. The hypothesis of this thesis therefore was that chemical-sensitive genes in embryos exposed in a conventional *Dar*T may indicate toxic impact of substances at sub-acute concentrations and thus enhance the sensitivity of the embryo toxicity test. Furthermore, unlike the conventional *Dar*T-endpoints, gene expression analysis will provide insights into mechanistic processes underlying toxicity. The 3,4-dichloroaniline (3,4-DCA), which is used as a reference compound in the *Dar*T, was selected as model chemical in this thesis.

In a first step, differentially expressed genes in embryos exposed to 3,4-DCA were identified by microarray technology and RT-PCR techniques. Six dose-dependent significant differentially expressed genes were identified. These genes were involved in

biotransformation pathways (*cyp1a*, *ahr2*), stress response (*nrf2, maft, ho-1)* and cell cycle control *(fzr1)*. Differential expression upon 3,4-DCA exposure was detected below the LOEC *(lowest observed effect concentration* = 6.2 μM*)* of survival or developmental disorders of the embryo test (0.78 μM and above). For the validation of stage specific sensitivity, genes were also analysed in post-hatched stages. Extension of exposure to post-hatched stages resulted in a differential expression at lower concentrations as for the embryonic stages, indicating an improved sensitivity due to stage-specific sensitivity or exposure time.

To confirm the adaptive function of the 3,4-DCA-sensitive genes, embryonic mRNA abundance was experimentally manipulated by knock down and overexpression. By injection of sense (mRNA) or antisense (siRNA) RNA in one-cell-stages of embryos, the transcript levels of genes were transiently enhanced or repressed in embryos exposed to 3,4-DCA. mRNA injection of the genes *cyp1a, ho-1* and *nrf2* reduced the number of embryos with 3,4-DCA-induced malformations. In contrast, siRNA injections for the same genes led to an increase in the severity and frequency of developmental disorders. The results clearly indicate the adaptive functions of the investigated genes or their corresponding proteins.

This study demonstrates that the analysis of chemical-sensitive gene expression shows the potential to increase the sensitivity of conventional toxicity tests. The analysis of gene expression also provides additional mechanistic information for toxic action, e.g. in the presented study, the involvement of Ah-receptor regulated pathways as an adaptive response. Furthermore, the presented data indicate that functional manipulations, using mRNA and siRNA-injection, are suitable to evaluate the role of differentially expressed genes for toxicity.

ZUSAMMENFASSUNG

Für die Zulassung von Chemikalien, Bioziden, Pestiziden und Medikamenten ist in Europa eine Umweltrisikoprüfung vorgeschrieben. Zur Vermeidung der Verbreitung potentiell gefährdender Substanzen werden verschiedene Toxizitätstest durchgeführt, darunter auch akute und chronische Fischtests. Als Konsequenz des neuen Zulassungsverfahrens der Europäischen Union (REACH = „registration, evaluation and authorisation of chemicals") wird innerhalb der Umweltrisikoabschätzung für die nächsten Jahre eine erhebliche Zunahme der Anzahl von Tierversuchen für die Substanztestung erwartet. Im Gegensatz dazu wächst der öffentliche Druck, Alternativen zu Tierexperimenten zu finden, um die Zahl von Tierversuchen zu reduzieren.

Nach geltenden EU-Richtlinien werden Experimente mit nicht-humanen Wirbeltierembryonen als schmerzfreie *in-vitro*-Methoden eingestuft und sind daher als Alternativen zu Tierversuchen akzeptiert. Als Ersatzmethode zum akuten Fischtest wird zum Beispiel der *Danio rerio* Embryotest (*Dar*T) eingesetzt, der in Deutschland im Rahmen der Untersuchung von Abwässern bereits in die gesetzliche Regelung integriert ist. Ersatzmethoden für chronische Fischtests existieren dagegen derzeit nicht. Im Hinblick auf eine solche Ersatzmethode war das Ziel dieser Arbeit die Erweiterung des Embryotest *Dar*T zu einem Gen-*Dar*T (Genexpression - *Danio rerio* Embryotest). Die Wirkung toxischer Substanzen entfaltet sich zunächst auf molekularer Ebene und kann Physiologie und Vitalität sowohl auf zellulärer, als auch auf organismischer Ebene beeinflussen. Genexpressionsanalysen erlauben es diese molekularen Vorgänge sichtbar zu machen, bevor phänotypische Veränderungen auftreten. Die dieser Arbeit zugrunde liegende Hypothese war daher, dass die Analyse chemikalien-abhängig exprimierter Gene in Embryonen des *Dar*T das toxische Potential einer Substanz bereits bei subakuten Belastungskonzentrationen anzeigen, und so die Sensitivität des konventionellen Embryotests erhöhen kann. Anders als die üblichen toxischen Endpunkte dieses Tests ermöglicht eine Untersuchung der Genexpression darüber hinaus Einblicke in zelluläre Mechanismen, die durch eine toxische Substanz beeinflusst werden. Als Modellchemikalie für diese Arbeit wurde

3,4-Dichloranilin (3,4-DCA) gewählt, das bereits als Referenzsubstanz im *Dar*T eingesetzt wird.

Über Mikroarray- und RT-PCR-Analysen wurden zunächst differentiell exprimierte Gene in Embryonen des Zebrabärblings identifiziert, die mit 3,4-DCA exponiert wurden. Sechs Konzentrations-abhängig exprimierte Gene wurden gefunden, die in Biotransformationsprozessen (*cyp1a*, *ahr2*), zellulären Stressantworten (*nrf2*, *maft*, *ho-1)* und in der Kontrolle des Zellzyklus *(fzr1)* involviert sind. Die differentielle Expression nach 3,4-DCA-Belastung konnte bereits unterhalb der LOEC *(lowest observed effect concentration* = 12.4 μM*)* für die toxischen Endpunkte Überlebensrate und Entwicklungsanomalien festgestellt werden (≥ 0.78 μM). Die Untersuchung der Genexpression in Larval- und Juvenilstadien zeigten, dass im Vergleich zum Embryo bereits bei geringeren Konzentrationen eine differentielle Expression beobachtet werden kann. Dies weist auf eine mögliche höhere Sensitivität späterer Entwicklungsstadien hin, könnte jedoch auch durch die verlängerte Expositionsdauer erklärt werden.

Zur Überprüfung der adaptiven Funktionen der 3,4-DCA-abhängig exprimierten Gene wurden mRNA-Mengen in Embryonen experimentell erhöht oder reduziert. Hierzu wurde mRNA bzw. siRNA in Ein-Zell-Stadien injiziert und der Einfluss auf die Toxizität von 3,4-DCA untersucht, Die Überexpression der Gene *cyp1a*, *ho-1* and *nrf2* führte zu einer verringerten Anzahl von durch 3,4-DCA hervorgerufenen Entwicklungsstörungen, während die Injektion von siRNA der gleichen Gene einen Anstieg der Entwicklungsstörungen zur Folge hatte. Diese Ergebnisse bestätigen somit die adaptiven Funktionen der untersuchten Gene bzw. ihrer Proteine bei einer Exposition mit 3,4-DCA.

Die hier vorgestellten Untersuchungen veranschaulichen das Potential Chemikalien-abhängig exprimierter Gene zur Abschätzung der Toxizität von Substanzen. Genexpressionsanalysen wie sie in dieser Arbeit durchgeführt wurden, sind geeignet die Aussagekraft des konventionellen Embryotests zu verbessern und vermitteln außerdem zusätzliche mechanistische Informationen, wie z.B. die Beteiligung von Ah-

Rezeptor-vermittelten Signalwegen als adaptive Antworten auf die Exposition. Durch Identifizierung weiterer Gene und Untersuchung zusätzlicher Chemikalien kann die Analyse der Genexpression in Zebrabärblingsembryonen zu einem Vorhersagemodell für chronische Toxizität entwickelt werden. Darüber hinaus zeigen die vorgestellten Ergebnisse, das funktionelle Genmanipulationen im Zebrabärblingsembryo zur Bewertung der Relevanz differentieller Genexpression für die Toxizität von Substanzen eingesetzt werden können.

CHAPTER I

INTRODUCTION

1.1 Scope of the study

For the registration of chemicals, pesticides, biocides and pharmaceuticals, acute and chronic fish tests are required for environmental risk assessment (Commission of the European Communities, 1967; ChemG, 1990; Commission of the European Communities, 1991, 1992, 1993a, b, 1994; PflSchG, 1998; CVMP/VICH, 2000, 2004; VICH, 2004; EMEA/CHMP, 2006). Due to the programme REACH (Registration, Evaluation and Authorisation of Chemicals, Commission of the European Communities, 2003), an increase of animal toxicity tests, including fish tests with up to 4 million more animals is expected for the first years (Bhogal, 2005). REACH was proposed by the EU commission in October 2003 as the new EU regulatory framework to improve the current state of human and environmental health. Substances entering the environment could exhibit a potential risk for both men and wild life. In REACH, only chemicals with a sufficient data set indicating no hazardous effects are allowed to enter the market. The required data sets depend on the production volume of the chemicals and apply also for already existing chemicals.

The increasing number of animal tests needed for chemical safety evaluation is incompatible with the societal demand for a refinement, reduction or even replacement of animal tests (The three R`s, Russel & Burch, 1959; Boghal, 2005). Based on this conflict, alternatives to conventional toxicity assessment of chemicals have to be developed. An *in vitro* alternative to acute fish toxicity, the *Danio rerio* toxicity test (*Dar*T, DIN 38 415-T6, Nagel, 2002) has been developed and implemented in German national regulations. For chronic fish tests, however, no alternative strategy could be established so far. The first research project working toward this goal was the Gene-*Dar*T project. The thesis presented here was embedded into Gene-*Dar*T[1].

[1]"Gene-*Dar*T - Development of a fish embryo test as alternative to prolonged and chronic fish tests: Analysis of toxic effects based on differential gene expression in the *Danio rerio* embryo test, project 1: Sensitive marker genes", funded by the German Ministry of Education and Research (BMBF) within the project framework "Biotechnology – alternatives to testing of animals" (FKZ 0313016)

The *Dar*T as an alternative to acute fish tests is based on observing phenomenological changes in an embryo. By using gene expression profiles as an additional endpoint, it may be possible to increase the sensitivity and to predict chronic toxicity. Gene expression is likely to be a very sensitive indicator of changes of cellular homeostasis. Exogenous substances may affect cell functions, reflected by a differential expression of genes. These alterations in gene expression can be either direct, e.g. by binding transcription factors, or indirect, e.g. as part of a general stress response. The aim of this thesis was the identification of differentially expressed genes and the characterisation of their expression in fish embryos exposed to the model compound 3,4-dichloroaniline (3,4-DCA). By gaining insight into the underlying mechanistic procedures and cellular events at the onset of toxicity, which could not be revealed by the classical endpoints (e.g. survival, developmental disorders) of the *Dar*T, these gene expression data are expected to improve the significance of the conventional toxicity test.

1.2 Alternative testing methods for fish toxicity tests

Fish are the most widely used non-mammalian vertebrates in animal toxicity testing for environmental risk assessment (Commission of the European Communities 1996). A number of different fish species are used as model organisms in ecotoxicology, e.g. the zebrafish (*Danio rerio*), the fathead minnow (*Pimephales promelas)*, the rainbow trout (*Oncorhynchus mykiss*), the golden orf (*Leuciscus idus*) and others. The performance of toxicity tests using one of the above listed model species is covered by several OECD guidelines (OECD 203, 210, 212, 215).

An *in vitro* alternative for fish tests – particularly acute tests – is the use of cell cultures (Schirmer, 2006). Due to the initiation of interactions between a toxic substance and an organism at the cellular level, it is assumed that cell culture systems can predict whole animal toxicity. They also represent a less expensive and less time consuming testing approach. Using cell cultures, a fast screening of potential toxic substances is possible. For instance, rainbow trout cells of liver, gills or gonads have been deployed as alternatives to toxicity tests with the golden orf or the rainbow trout (Halder & Ahne,

1990; Castaño *et al.*, 2003; Dayeh *et al.*, 2002). However, due to their relatively low sensitivity, fish cells have not yet been implemented in regulatory testing in ecotoxicology. A reason for the low sensitivity could be the undifferentiated stage of many continuous cell lines and the loss of specific features like receptors or other enzymes over the years. Furthermore, experimental conditions, such as the use of serum with an acssociated reduction of freely available experimental exposure concentrations sets limits to the applicability of cell culture systems in toxicology (Schirmer, 2006).

The use of embryonic stages represents another *in vitro* approach for the assessment of toxicity. According to the German animal protection act and an EU directive (European directive 86/609/EEC, 1986), investigations using vertebrate embryos are considered pain-free *in vitro* methods and are therefore accepted as alternative tests. Nagel (2002) developed an alternative to the acute fish toxicity test (OECD guideline 203) using zebrafish embryos. In the so-called *Danio rerio* embryo test (*Dar*T), zebrafish embryos are exposed to the test compound from fertilisation to 48-50 hours of development. Different acute and sub-acute toxicity endpoints, comprising survival and developmental disorders, are examined. The sensitivity of the embryo test is similar to the acute fish test and its reproducibility has been validated by a Round Robin test (Nagel, 2002). In 2005 the *Dar*T, also called fish egg test (DIN 38415-6, 2001), has been implemented in the German waste water dues law "Wasserabgabengesetz" to replace the golden orf in waste water testing (Bundesgesetzblatt, 2005).

1.3 The model organism zebrafish (*Danio rerio*)

The zebrafish (*Danio rerio, Cyprinidae*) has a long tradition of application in developmental biology, pharmaceutical and environmental toxicology. This teleost has emerged as one of the most important models for molecular genetics as well as neurobiological studies. The zebrafish features different advantages, particularly with respect to its embryonic development. Eggs develop externally and fertilisation time can be manipulated by modifying the spawning time via the light-dark cycle. Several 100 fertilised eggs can be obtained from a single mating pair. The synchronous

development and the transparency of eggs facilitate the study of development including the formation of internal organs (Linney *et al.*, 2004). The embryonic development is relatively short and embryos hatch within three days. The yolk is only slowly digested so that no food needs to be provided during early life stages. Working with zebrafish also is advantageous because the zebrafish genome is now nearly fully sequenced. For many genes expression patterns are available and functional data exist because of a large number of mutants and studies on transient gene manipulations.

Due to the advantages for both (eco-) toxicology and developmental biology, it is apparent, that zebrafish embryos could also be successfully utilised in the field of (eco)toxicogenomics. To assess the potential toxicological impact of substances on zebrafish embryonic development, a set of different molecular tools, commonly used to discover mechanisms in developmental biology, can be deployed:

- Microarray technology can be used to screen for a whole set of genes, of which the expression is influenced by exposure to toxicants.
- In situ hybridisation and transgenic reporter strains can help to analyse the spatio-temporal expression of genes and to indicate organ related functions.
- Repression or overexpression of mRNA abundance can be deployed to study gene function in order to clarify the relevance of genes involved in the response to a toxic compound.

1.4 The model substance 3,4-dichloroaniline (3,4-DCA)

In this thesis 3,4-dichloroaniline (3,4-DCA, CAS number 95—76-1, molecular weight 162 g/mol) was used as a model compound to identify and characterise genes which are differentially expressed in zebrafish embryos exposed to toxic chemicals. 3,4-DCA consists of an aromatic ring, an amino group and two chlorines substitutes at position 3 and 4 (figure 1.1).

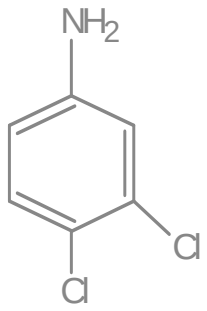

Figure 1.1 Chemical structure of the model compound 3,4-dichloroaniline investigated in this thesis

3,4-DCA is an intermediate of the production of herbicides, bactericides and dyes. The mayor release of 3,4-DCA into the environment occurs during the degradation of phenyl amide herbicides, like diuron, propanil and linuron, in soil and water. These herbicides are very stable to hydrolysis and photolysis. They are broken down rapidly by aerobe and anaerobe microbiological degradation (Giacomazzi & Cochet, 2004). The bacterial break down of diuron (*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethyl-urea) is proposed to involve the formation of isocyanides and isocyanic acids and the final hydrolysis to amines, carbon dioxide and subsequently 3,4-DCA. 3,4-DCA has a higher toxicity and is detected more frequently in environmental samples than its parent compound (Giacomazzi & Cochet, 2004; Claver *et al,* 2006). 3,4-DCA itself is neither readily biodegradable nor hydrolysable. Like its parent compounds, 3,4-DCA is only slowly degraded by light in water (Miller *et al.*, 1980). Thus, 3,4-DCA is frequently found in soils and water (Claver *et al*., 2006).

3,4-DCA is classified as a highly toxic, environmental hazardous substance. Toxicity was demonstrated for many organisms, including protozoa (Tixier *et al.*, 2001), algae (Girling *et al.*, 2000), insects (Sosak-Swiderska *et al.*, 1998), fish (Ensenbach *et al.*, 1996) and mammals (Valentovic *et al.*, 1997). Toxic effects upon dermal contact and inhalation involve the formation of methaemoglobin. In rats, kidney, liver and urinary bladder have been identified as main target tissues for toxicity (Valentovic *et al.*, 1997).

Very little is known about the molecular effects of 3,4-DCA, but an induction of HSP70 protein has been observed in human cells (Ait-Aissa *et al.,* 2000). Quantitative structure activity analyses have indicated that toxicity of 3,4-DCA can be described by the polar narcosis mode of action (Arnold *et al.,* 1990). Thus, it may resemble many other toxic chemicals that are anticipated to act by an unspecific so-called baseline toxicity (Escher & Schwarzenbach, 2002). The chronic toxicity of 3,4-DCA has been analysed in a life cycle test and in early life stages of zebrafish. Based on this, 3,4-DCA was used as model compound for fish toxicity tests (Nagel *et al.,* 1991; Schäfers *et al.,* 1993). It is also the reference compound for the standard *Dar*T protocol (DIN 38415-6 2001).

1.5 Potential applications of gene expression in alternative testing strategies

Gene expression is likely to represent a sensitive endpoint. Effects of chemicals are primarily manifested by interacting with molecular processes that lead directly or indirectly to alterations in mRNA abundance. These interactions can occur at different levels. A compound could interfere with membrane integrity, bind to or react with proteins, lipids and DNA. Finally, important cellular functions may be disturbed that could lead to the death of the cell or the organism. The molecular changes accompanying these processes can be related to either adaptive processes or to the mediation of toxic effects (Girardot *et al.,* 2004). They have been shown to indicate harmful effects of chemicals in cases where by classical toxicological endpoints no obvious effects have been detected (Heinloth *et al.,* 2004). Changes in gene expression levels by chemicals could result from an alteration in cellular homeostasis such as the interference with cellular components at various levels. Thus, gene expression analysis in *Dar*T is likely to reveal additional mechanistic information, which could not be obtained by the classical toxic endpoints of the embryo test. By analysing expression of toxicant-sensitive genes, sensitivity of the embryo test may increase and a correlation and prediction of chronic toxicity might be possible.

1.6 Identification of toxicant-sensitive genes

Various genes (or their corresponding proteins) involved in biotransformation, drug

resistance, apoptosis and other processes are known as (possible) candidates for toxicant-sensitive expression:

Biotransformation

Defence against the potential harm of xenobiotics or endogenous toxic metabolites is mainly executed by so-called xenobiotic metabolizing enzymes (XME). In many cases, exposure to xenobiotics induces the expression of XME genes with subsequent increased enzyme activity. XMEs are classified according to their function in phase 1 (hydroxylation reaction), phase 2 (conjugation reaction) and phase 3 (excretion) biotransformation enzymes. Prominent examples for xenobiotics which affect transcription of XMEs are halogenated and non halogenated polycyclic hydrocarbons (hPAH, e.g. dioxin and PAHs, e.g. benzo(a)pyrene). These substances are known to affect gene expression patterns of phase 1 (cytochromes P450 (CYP)) and phase 2 enzymes (UGT, GST) via binding to the aryl hydrocarbon receptor (AHR).

The aryl hydrocarbon receptor is one of several vertebrate intracellular receptors, which activate expression of a battery of genes involved in biotransformation and which often are transcriptionally autoregulated. Thus, activation of the receptor also causes induction of its gene. Without ligand activation, most of these receptors are bound to chaperones in the cytosol. Upon activation by binding of a ligand, they are translocated to the nucleus, bind to specific recognition sites of gene promoters (so-called xenobiotic response elements (XRE, for AHR: ARE) and subsequently induce the transcription rates of biotransformation enzymes (Denison & Nagy, 2003).

In a classical phase 1 reaction, a drug metabolizing enzyme like cytochrome P450 catalyses the addition of a hydroxyl group. This facilitates conjugation reactions of phase 2 enzymes (Rushmore & Kong, 2002). Phase 2 reactions are conjugation reactions, converting the substrate into a more water soluble compound and enhancing excretion via bile or the urine. Phase 2 xenobiotic metabolizing enzymes include sulfotransferases (SULT), UDP-glucuronosyltransferases (UGT), NAD(P)H:quinone oxidoreductases (NQO), epoxide hydrolases (EPH), N-acetyl transferases (NAT) and glutathione S-transferases (GST) (Rushmore & Kong, 2002). Transcription of phase 2

genes is regulated by transcription factors which are activated by AHR mediated signalling, mitogen-activated protein kinase pathways (MAPK) or other transduction processes. MAPK pathways can serve as signal converters, which translate a wide range of external stimuli, including xenobiotics, into intracellular responses through serial phosphorylation cascades (Rushmore & Kong, 2002). The basic leucine zipper NF-E2 p45 related factors 1 and 2 (Nuclear factor E2 p45-related factors 1 and 2, NRF1, NRF2; Itoh *et al.*, 1997) are transcription factors involved in the MAP-kinase pathway.

Intermediates of biotransformation may be even more toxic than their parent compounds and may cause cell damage by interaction with proteins, RNA or DNA (Vermeulen, 1996). For instance the CYP1A mediated oxidative metabolism of benzo(a)pyrene causes the formation of reactive epoxides which are able to produce DNA adducts. These are suspected to be one important factor in the evolvement of lung cancer associated with cigarette smoking (Yang *et al.*, 1977). Thus, induced gene expression of xenobiotic metabolizing enzymes does not necessarily lead to a protection against the xenobiotic compound.

Multi xenobiotic resistance

In addition to phase 1 and phase 2, a third phase can be defined, describing the active elimination of xenobiotics out of a cell. Antiporters like p-glycoproteins or multi drug resistance proteins (MDR) are energy-dependent efflux pumps, which selectively pump out substances and thereby decrease intracellular concentrations of potential hazardous compounds (Bard, 2000). Proteins which mediate chemical resistance of cells, are e.g. major vault protein (MVP) or multidrug resistance-related protein (MRP). MDRs might also be transcriptionally activated upon chemical exposure (Luedeking & Koehler, 2004).

Oxidative stress

Various proteins are involved in cytoprotective processes upon oxidative stress. These proteins are likely to be transcriptionally activated by stimuli of various exogenous inducers such as heavy metals, endotoxins, heat shock, inflammatory cytokines, prostaglandins and other agents causing oxidative stress. Involved enzymes are for

instance heme oxygenase 1 (HO-1), catalase or cu/zn-superoxid-dismutase (SOD1) (Choi & Alam, 1996; Yin *et al.*, 1998). Other genes affected by oxidative stress are oxygen dependent transcription factors, like the hypoxia inducible factor 1 (HIF1), which targets genes especially related to angiogenesis, cell proliferation/survival and glucose/iron metabolism (Lancaster *et al.*, 2004).

Stress response

Prominent proteins involved in the cellular response to heat or hypoxia are heat shock proteins (HSP). Most of the HSPs possess chaperonin function, i.e. they control correct folding of proteins (HSP70, HSP47, BIP) (Lele *et al.*, 1997, Ton *et al.*, 2003). HSPs are transcriptionally regulated by heat shock factors (HSFs), which bind as homotrimers to heat shock promoter elements (HSEs) in order to activate transcription. Trimer formation is induced by non-native, misfolded or aggregated proteins, which are likely to occur upon heat shock, hypoxia, altered pH, but also upon chemical impact (Morimoto, 1998). The HSF cycle is regulated by a negative feedback loop via the binding of the heat shock proteins HSP70 and HDJ1, but also by binding of the heat shock factor binding protein (HSBP). The trimers dissociate to monomers which remain inactive via transient interaction with hsp70 and hsp90 in the nucleus or cytoplasm (Morimoto, 1998).

DNA repair

Eukaryotic DNA mismatch repair (MMR) proteins like MSH 2 and 6 are protein complexes which recognize and repair base/base mismatches, insertion and deletion loops, as well as other types of DNA damage (Fischel, 1998; Kolodner & Marsischisky, 1999). Another DNA repair system consists of RAD proteins which are induced upon DNA double strand breaks (Ivanov & Haber, 1997). DNA repair proteins support the prevention of mutagenesis, cancer and the induction of genotoxicity and apoptosis. Activation of DNA repair proteins is mainly performed posttranslational upon exposure to agents causing DNA damage (Belloni *et al.*, 1999; Peterson-Roth *et al.*, 2005). However, there are also reports of chemical-induced transcriptional upregulation of mismatch repair proteins (Humbert *et al.*, 2003).

Apoptosis

Apoptosis (programmed cell death) plays an important role in normal differentiation, but can also be initiated by the impact of chemicals (Ellis *et al*., 1991; Steller, 1995). Apoptosis involves differential expression of various genes whose transcription might also be altered upon chemical-induced apoptosis.

Cytosolic Ca^{2+} is an intracellular signalling ion and a proapoptotic second messenger, modulating signal transduction pathways by phosphorylation/dephosphorylation of proteins (Nicotera *et al*., 1990; Pan *et al*., 2001). Reactive oxygen species (ROS) or reactive nitrogen species (RNS) can cause influx of Ca^{2+} from the extracellular matrix, the endoplasmatic reticulum or the sarcoplasmatic reticulum into the cytoplasm. Subsequently, Ca^{2+} influx into nuclei and mitochondria is enhanced. Rising Ca^{2+} concentrations in mitochondria disturb normal metabolism and finally cause cell death (Ermak & Davies, 2002). In the nuclei, Ca^{2+} influences gene expression leading to the regulation of apoptosis-specific enzymes (Ermak & Davies, 2002). It can be anticipated that many toxic compounds, particularly those interfering with membranes, affect Ca^{2+} levels and lead to differential expression of genes involved in apoptosis.

The tumour suppressor transcription factor P53 is activated upon DNA damage or other stressors and may mediate cell cycle arrest or apoptosis by induction of proapoptotic enzymes (Robles *et al*., 2001). Proapoptotic enzymes which have been shown to be induced by exposure to chemicals are members of the BCL family including BAD, BAX and BIK (Hsieh *et al*., 2003, Fernandez *et al*., 2003; Hur *et al*., 2004). Examples for transcriptionally regulated proteins during cadmium-induced apoptotic processes are the tumour suppressor gene P53, the Cdk kinase inhibitor P21, the proapototic BAX and the BAX-antagonist BCL2 (Fernandez *et al*., 2003).

Apart from the processes described above, the expression of other currently unknown genes of divergent cellular function might be affected. To identify these genes and to monitor the underlying molecular mechanisms of the response to xenobiotic exposure, gene expressing profiling using microarray technology is a valuable tool. Microarrays can be deployed to screen whole transcriptomes and to indicate which signal

transduction pathways are involved in the cellular response to chemical exposure.

1.7 Identification of differentially expressed genes by microarray technology

In the last decade, microarray technology was developed as a novel approach to assess genome-wide changes in gene expression. Traditional techniques like RT-PCR and Northern blotting of mRNA can only evaluate the alterations in expression of a limited number of genes. In contrast, the invention of DNA microarray technology enables the simultaneous investigation of 10000s of genes or whole transcriptomes (Brown & Botstein, 1999). For this technique, detailed information for the genome of the target organism is advantageous. Therefore, gene expression profiling using microarrays is in particular applicable for model organisms with a well characterised genome. The development and first application of a DNA microarray was reported by Schena *et al.* in 1995. At the beginning it was primarily applied for diagnostic purposes in human medicine. Since then, microarray technology has been applied to many different fields. One of these fields is toxicogenomics. In the last years, microarray technology has been deployed to assess the toxicological response to environmental contaminants as well as the pharmaceutical response to chemotherapeutical agents (Nuwaysir *et al.*, 1999).

When applying microarrays it must be considered that many proteins are regulated posttranslational. These regulations would not be detected by gene expression analysis. However, they might be reflected by genes controlled via a corresponding signal transduction pathway. Alternatively, to detect protein regulations and alterations, proteome analysis could be used. However, at the current state of technology, microarray technology is more advanced and better suited for high trough-put analysis.

The microarray technique is based on the hybridisation of complementary nucleotide sequences. Dye-labelled cDNA or antisense RNA (aRNA) is bound to complementary DNA-fragments, immobilised on a slide surface. These complementary fragments, which can be either oligonucleotides or cDNA sequences, are covalently bound to the surface of a pre-treated glass slide (e.g. poly-L-lysine, epoxy or aminosilane slides) or a

nylon membrane. Printing of the fragments is performed by a spotter device. cDNA probes of treated as well as control samples are prepared by reverse transcription from mRNA. During cDNA synthesis these probes can be either directly linked to the fluorescent dyes by incorporation of dye-coupled deoxynucleotide-triphosphates or by incorporation of modified aminoallyl-UTP (5-(3-aminoallyl)-2`deoxyuridine-5`-triphosphate, AA-dUTP) and subsequent covalent coupling of fluorescent dyes to the aminoallyl molecules. For treated and control samples, cDNA is linked to different dyes (e.g. cyanine 3 and cyanine 5). The cDNA probes are combined for hybridisation on the array (figure 1.2). Alternatively, aRNA can be deployed for hybridisation. The aRNA is synthesised by *in vitro* transcription of a double-stranded cDNA. Aminoallyl-UTP is incorporated during *in vitro* transcription.

After hybridisation, the array is scanned at the excitation wave lengths of the labelled dyes. Due to the differences in the abundance of the bound cDNA/aRNA probes of both samples, different fluorescence intensities for treatments and controls can be assessed. Fluorescent intensity can be correlated to the relative mRNA abundance of the sample (Brown & Botstein, 1999; Sterrenburg *et al.*, 2002). The ratio of intensities of both fluorescent dyes is an indication for the induction or the repression of a gene under a certain condition in comparison to the control situation (figure 1.2).

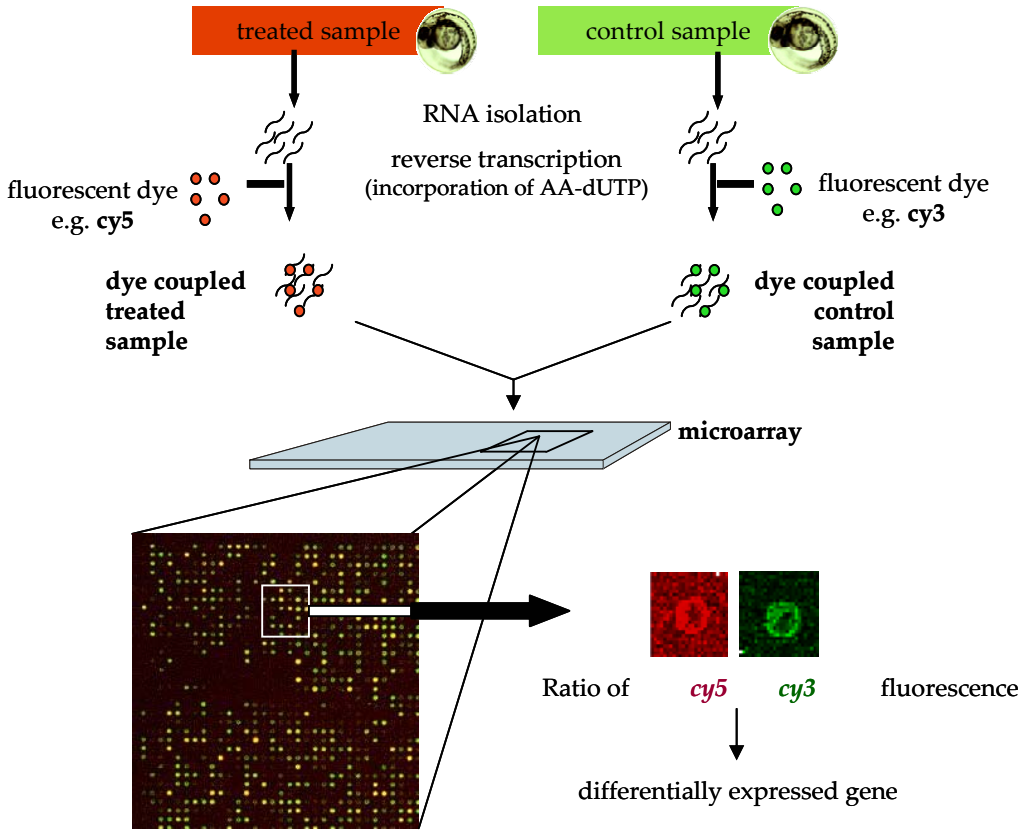

Figure 1.2 Work flow scheme of the microarray experiments conducted in this thesis. cDNA of treated samples and control samples were labelled with different fluorescent dyes (cy3 or cy5, respectively) via binding to aminoallyl-dUTP. Combined samples were hybridised to a 14 k zebrafish oligonucleotide array.

In this thesis a zebrafish specific oligonucleotide microarray, consisting of more than 14000 genes was used to identify genes with altered expression upon exposure to the model compound. Since the genome of the zebrafish is actually expected to comprise between 26600 and 48200 genes, this array represents 29 to 53 % of the zebrafish transcriptome (Ensemble zebrafish assembly version 6, http://www.ensembl.org/Danio_rerio/index.html).

Microarrays are not only a valuable new tool in (eco-) toxicology for a fast and genome wide screening of chemical-sensitive genes. They furthermore help to understand the underlying molecular mechanism of toxic or adaptive effects. This new possibility of gene expression profiling improves and refines the risk assessment of chemicals. The prediction of phenotypical consequences from gene expression profiles will be one of

future challenges of toxicogenomics using microarray technology (Nuwaysir *et al.*, 1999).

1.8 Analysis of gene function

Traditionally, studies of gene function were performed with mutants. Knockout mutants of various genes are available for model organisms like mouse and zebrafish. Alternatively, the function of genes can be assessed by transient loss- and gain of function studies avoiding the time-consuming and laborious process to establish a mutant. This approach is widely applied in embryos as well as in cell culture systems. For the enhancement of mRNA abundance, the corresponding cDNA is cloned into bacterial overexpression vectors. The mRNA of the gene is transcribed *in vitro*, purified from the vector and injected. Prominent applications of overexpression studies are for instance the analysis of functions of *pax* genes during embryonic eye development (e.g. Halder *et al.*, 1995; Onuma *et al.*, 2002) and investigations concerning the relevance of *Wnt* signalling in the formation of boundaries of the central nervous system (reviewed in Wodarz & Nusse, 1998).

Vice versa, gene expression can be transiently repressed. In order to induce a loss of function, antisense nucleotides specifically inhibiting gene function are introduced into the cell or organism. One possibility to efficiently knock down gene expression during embryonic development is the utilisation of so-called morpholino antisense oligonucleotides (MO). MOs specifically inhibit gene expression by binding and inactivating RNA sequences, reducing their translation rates and thus, disturbing gene function. Instead of ribose or deoxyribose, the morpholino molecular structure consists of a 6-membered morpholine ring, which is linked by non-ionic phosphorodiamidates instead of anionic phosphodiesters (www.genetools.com; Heasman, 2002). Morpholino antisense oligonucleotides have already been successfully applied in zebrafish in the field of toxicogenomics. For instance, Teraoka and colleagues (2003) found an inhibition of TCDD (2,3,7,8-tetrachloro-[p]-dibenzodioxin)-developmental toxicity in embryos upon knock down of AHR2 and CYP1A using MOs. Using AHR2 morpholinos, both TCDD- and phenylthiourea-mediated CYP1A-activity was found to

be modulated by the same AHR signalling pathway, while ARNT2 morpholinos revealed that ARNT2 is not required for the formation of TCDD toxicity (Wang *et al.*, 2004; Prasch *et al.*, 2003; Prasch *et al.*, 2004).

MOs are designed against the 5`upstream untranslated region, where they bind to the translation start site AUG or interfere with the progression of the initiation complex, which subsequently fails to reach the translation start. The limitation of the mopholino approach is that repression occurs at the level of translation. This means that the effect of MOs is more difficult to control. Here, control would best be archieved by observing a down-regulation of the target protein, e.g. by immune or protein activity assays.

An alternative technique, which has been applied in this thesis, is the method of short interfering RNA (RNA interference, RNAi). Doubled-stranded RNA (dsRNA) interference is a natural occurring process, which has recently been successfully adopted to study gene function. In eukaryotes, RNAi is involved in the cellular defence against viral invasion, transposon expansion or post-transcriptional gene silencing (Tuschl, 2001; Hannon, 2002). In response to these pathogens, the host cell initiates the RNAi reaction: Double-stranded RNA is cleaved into 19 – 25 nt long, small interfering RNA (siRNA) with 3´dinucleotide overhangs by the cytoplasmic RNAse III enzyme DICER. The siRNA is incorporated into the RNA-induced silencing complex (RISC), which is then activated by ATP to unwind the duplex. The two strands can now specifically and independently target and identify the mRNA, whereupon the RISC complex mediates the cleavage of the mRNA sequence (Tuschl *et al.*, 1999, Elbashir *et al.*, 2001a). The aberrant mRNA is identified by the cell and is subsequently degraded (figure 1.3).

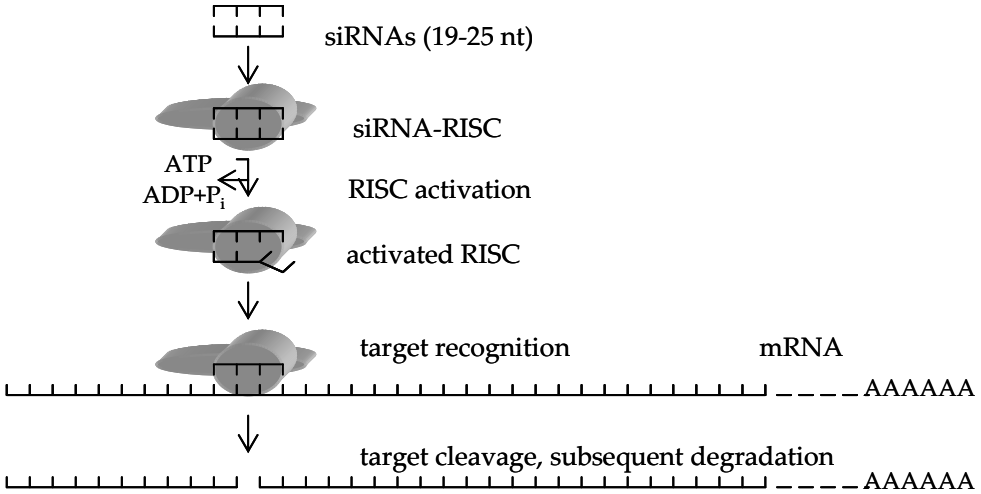

Figure 1.3 Principle of RNA interference: The siRNA is incorporated into RISC (RNA-induced silencing complex), which is activated by ATP. The siRNA-RISC complex binds the complementary sequence on the target mRNA. RISC is mediating mRNA cleavage and in turn gene silencing occurs (changed after „RNA interference – technical reference & application guide", Dharmacon, Inc, 2004).

RNAi was detected in 1990 in plant and fungi (Jorgensen, 1990). In 1999 it was for the first time applied in functional gene studies to sequence-specifically suppress target mRNA (Fire, 1999). However, first attempts of mRNA silencing in vertebrates were of limited success. In mammalian cells transfected with long dsRNA, the interferon response results in a general inhibition of protein synthesis, which might lead to cell death (Baglioni, 1979). This response represents an unspecific cellular defence and eliminates the utilisation of long dsRNA to silence mRNA in mammalian cells. In addition to unspecific responses in mammalian cells, Oates *et al.* (2000) found unspecific effects in zebrafish embryos upon injection with double-stranded RNA. Thus, dsRNA-mediated silencing was limited to plants, *C. elegans* or *D. melanogaster*.

To overcome the limitations of RNAi, synthetic, 19 to 25 nt short dsRNA sequences were developed to specifically silence target mRNA in vertebrate cell lines and zygotes of different species (Caplen *et al.*, 2001; Elbashir *et al.*, 2001b; Yang *et al.*, 2002). Synthetic siRNA is able to mimic the silencing effect of natural siRNA at nanomolar concentrations, persisting for several cell divisions up to several days. Up to date, this method was mainly used in cell cultures or deployed for functional manipulation of genes during *Drosophila* embryonic development (Harborth, 2001; Caplen *et al.*, 2000;

Berns *et al.*, 2004; Knorr-Wittmann *et al.*, 2005; Saito *et al.*, 2006; Yang *et al.*, 2005, Wagh *et al.*, 2006). Only a few publications refer to the application of RNAi in zebrafish. In 2004, Dodd *et al.* demonstrated the success of siRNA to knock down the expression of the zebrafish *dystrophin* gene. In 2005, it was shown by Liu *et al.* that injection of *no tail* siRNA lead to the same phenotype observed in mutants.

If compared to the morpholino approach, the application of siRNA for gene silencing exhibits several advantages. Suppression occurs at a transcriptional level, thus, the efficient repression of the mRNA level can easily be confirmed using RT-PCR or in situ hybridisation. Furthermore, RNA silencing is less cost intensive. siRNA harbours effective and reproducible silencing effects already at non-toxic concentrations, which is often not the case for other knock down applications, e.g. double-stranded antisense DNA (Heasman, 2002). Thus, siRNA is particularly of interest for studying gene expression in toxicology.

1.9 Specific objectives of the thesis

The objectives of this study were:

- the identification of toxicant-sensitive genes in zebrafish early development using the model compound 3,4-dichloroaniline (3,4-DCA) and
- the characterisation of the function of the genes for either mediating toxicity or adapting to toxicity of 3,4-DCA.

In order to archieve these goals, exposure experiments using 3,4-DCA were carried out according to the standardized *Dar*T protocol (DIN 38415-6 2001). Microarray and RT-PCR techniques were performed to screen for 3,4-DCA-dependently expressed genes. In order to unravel the implications of differentially expressed genes in the onset of toxicity, gain- and loss studies using mRNA overexpression and siRNA knock down techniques were applied. The experiments were designed to demonstrate whether gene expression analysis can be deployed as an additional endpoint in the *Dar*T and whether - unlike the conventional toxic endpoints – it could provide information about

molecular mechanisms and cellular processes in response to the toxicant. It was furthermore tested whether gene expression enhances the sensitivity of the *Dar*T and principally extends its predictive value to chronic toxicity.

CHAPTER II

MATERIALS & METHODS

2.1 *Danio rerio* embryo test (*Dar*T)

The zebrafish wildtype strain WiK (wild type Kalkutta) was obtained from the Max-Planck-Institute of Developmental Biology in Tuebingen (Germany) originating from a wild catch in India and then kept as close stock of several sublines from single-pair matings (Rauch *et al.*, 1997). Fish were cultured at 26 ± 1 °C at a 14:10 hours light:dark cycle and were fed daily, once with *Artemia* and twice with commercial flake food (Tetra, Melle, Germany). Collection of eggs and exposure of embryos were performed according to standard protocols (Schulte & Nagel 1994; Nagel, 1998).

Spawning and fertilisation of zebrafish eggs was triggered by the light:dark cycle and glass trays covered by nets, in order to mimic natural hiding places. Embryos were collected from the glass trays of the spawning zebrafish culture. Staging of embryos was performed according to Kimmel *et al.* (2005).

Exposure was performed in glass petri dishes (diameter of 7 cm for up to 30 embryos) in a volume of 10 ml. The test medium consisted of 2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.75 mM $NaHCO_3$ and 0.08 mM KCl. Different concentrations of the test chemicals were added to the test medium by aqueous stock solutions in water (3,4-dichloroaniline, 3,4-DCA) or DMSO stock solutions (β-naphthoflavone). Embryos were exposed for 48 hours to 0.78, 1.54, 3.1, 6.2 or 12.4 µM of 3,4-DCA (Fluka Chemie AG, Buchs, Germany; purity 99 %, stock solution in water: 308.5 µM as confirmed by GC-MS, see 2.3).

Exposure started at 2 hpf (hours post fertilisation) and was continued for 48 hours. 30 embryos were exposed for each concentration. At 24 and 48 hpf, embryos were examined for developmental disorders and viability using a dissection microscope. According to the OECD guideline for testing of chemicals (OECD DRAFT,1998, Fish embryo toxicity test with the zebrafish *Danio rerio*), lethality was indicated by the coagulation of embryos, incomplete formation of all 20 somites, a non-detached tail and the missing of heart beat. As sub-lethal endpoints, abnormalities in the formation of the lens placode, in spontaneous myotomal contractions, in blood circulation, in pigmentation and in the appearance of oedemata were considered.

Lethal concentrations (LC_x) and effect concentrations (EC_x) of 3,4-DCA were calculated by a Probit transformation (linear maximal Likelihood regression). Due to correspondence of data with normal distribution of greater than 50% (R/S-test), NOEC (*no observed effect concentration*) and LOEC (*lowest observed effect concentration*) were determined by the parametric Dunnett's test ($p< 0.05$, ToxRat software, ToxRat Solutions GmbH, Alsdorf, Germany). At the end of the exposure (at 50 hpf), embryos were collected, shock frozen in liquid nitrogen and stored at -80°C until RNA isolation. Samples were used for gene expression analysis using microarray technology or for RT-PCR techniques. In order to avoid that morphological disorders affected gene expression patterns, only embryos without any visible developmental abnormalities such as shortened tail, reduced head size and others were selected for RNA isolation of microarray samples.

2.2 Early life stage test (ELST) using *Danio rerio*

The ELST was performed according to OECD guideline 210 at the Institute of Hydrobiology, University of Dresden by Dr. M. Tillmann. 210 zebrafish embryos were exposed to each of five different concentrations of 3,4-DCA (0.11 µM, 0.22 µM, 0.46 µM, 0.93 µM and 1.85 µM) in a flow through system from 2 hpf until 30 dpf. The experiment was replicated twice; mortality and developmental aberrations (oedemata, pigmentation etc.) were observed daily. At 5 and 30 dpf, samples of larvae or juvenile fish, respectively, were collected for gene expression analysis. Samples were shock frozen in liquid nitrogen and stored at -80°C.

LC_x and EC_x of 3,4-DCA were determined by a Probit transformation (linear maximal Likelihood regression). Due to the normal distribution of data (> 50 %, R/S-test), NOEC and LOEC were calculated by parametric Dunnett's test ($p< 0.05$, ToxRat software).

2.3 Chemical analysis of 3,4-dichloroaniline

For the embryo test, 3,4-DCA was analysed with GC-MS (gas chromatography – mass spectrometry) and a SIM (single ion mode) detector (HP 6890 GC system with 5973

mass selective detector, Agilent Technologies, Böblingen, Germany) measured by the department of analytics of the UFZ-Centre for Environmental Research Leipzig-Halle GmbH (Leipzig, Germany). 3,4-DCA concentrations in the early life stage test (ELST) were measured by DVGW Technologiezentrum Wasser (Karlsruhe, Germany). GC/MS analysis was performed with an Autosystem XL gas chromatograph and an AutoMass Gold Detector (both Perkin Elmer, Rodgau, Germany) using a Zebron-5 column (30 m x 0,25 mm x 0,25 µm, Phenomenex, Aschaffenburg, Germany).

In the embryo test, chemical analyses at concentrations below 12.4 µM reflected the nominal concentrations and indicated neither degradation of the chemicals nor absorption to the glass petri dishes used for the exposure. Measured test concentrations at the end of the exposure after 48 hours were 0.002 (nominal 0), 3.4 (nominal 3.1) and 6.2 (nominal 6.2) µM 3,4-DCA. Only at 12.4 µM 3,4-DCA – the highest concentration tested – a decrease of the concentration to below 50% of the nominal concentration (measured 5.5 µM) did occur in repeated experiments after 48 h of exposure. In the ELST, the mean measured concentrations of 3,4-DCA were between 10 and 143% of the nominal concentration (at day 14: 0.00055 (nominal 0), 0.011 (nominal 0.11), 0.27 (nominal 0.46), 0.38 (nominal 0.93), 3.2 (nominal 3.1) µM 3,4-DCA). Toxicity parameters LC_{50}, EC_{50}, EC_{10}, LC_{10} and NOEC and LOEC were calculated based on nominal concentrations because measured concentrations were close to the nominal concentrations in most of the treatments.

2.4 RNA Extraction

Total RNA was extracted from a pool of 30 (for RT-PCR analysis) or 100 (for microarray analysis) 50 hpf-old homogenised zebrafish embryos with Trizol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Likewise, total RNA was extracted from a pool of 65 larvae or 3 juvenile fish originating from the early life stage test. RNA samples were stored at -80°C.

2.5 Conventional and quantitative RT-PCR

2.5.1 Procedure and data acquisition

Contaminations of genomic DNA were removed by treatment of 2 µg total RNA with 1 unit of DNAse I (Roche, Grenzach, Germany) for 15 min at 25° C in a reaction volume of 10 µl. cDNA was synthesised from DNAse treated RNA using the RevAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Primers were designed using the computer program Primer3 (Rozen & Skaletsky 1998) and obtained from Invitrogen.

For conventional RT-PCR, target genes and the reference genes *β-actin* or *cyclophilin A*[2] were amplified from 1 µl of cDNA using 1 unit of Taq Polymerase (Promega, Mannheim, Germany), 50 mM TRIS-HCl (pH 9.0, Serva, Heidelberg, Germany), 1.5 mM $MgCl_2$ (Sigma, Steinheim, Germany), 15 mM $(NH_4)_2SO_4$ (Sigma), 0.1% (v/v) Triton-X 100 (Merck, Darmstadt, Germany), 0.2 mM dNTP mix (MBI Fermentas) and 0.4 µM of each primer in a 50 µl reaction volume. The number of cycles was adjusted to obtain amplified DNA during the exponential phase of the reaction. Annealing was performed at 55°C. PCR-fragments were analysed by agarose gel electrophoresis (1.5 % w/v agarose) and ethidium bromide staining (0.005% w/v). mRNA abundance was evaluated by either visual comparison of band intensity or densitiometric analysis (i.e. semiquantitative RT-PCR). Densitiometric analysis was performed from three different experiments. The image analysis software ImageJ (Version 1.33u, freely available at http://rsb.info.nih.gov/ij/) was used to calculate expression levels from relative band intensities of agarose gels. Intensities of the transcripts of genes of interest were normalised to the band intensities of the transcript of the reference gene before further data analysis and statistics (see 2.5.2).

[2] cyclophilin A is a cytosolic protein, exhibiting a peptidyl-prolyl-cis-trans isomerase activity (PPiase), which facilitates protein folding of proline-consisting proteins in order to support confirmation changes of signal transduction processes. This gene is ubiquitious expressed in all cells and thus an appropriate constitutive control for PCR.

Quantitative RT-PCR was carried out in a reaction volume of 25 µl containing 1 µl of cDNA, 1x qPCR buffer (Taq PCR Core Kit, Qiagen, Hilden, Germany), 3.5 mM $MgCl_2$, 0.2 mM dNTP mix (Taq PCR Core Kit, Qiagen,), 0.02 µM FITC (fluorescein isothiocyanate, Bio-Rad, Munich, Germany), 0.05 µl SYBR green I (100x dilution in DMSO; Sigma), 0.5 U Taq DNA polymerase (Taq PCR Core Kit, Qiagen) and 0.1 µM of each primer. Amplifications were performed in 96-well plates (Peqlab, Erlangen, Germany) in the iCycler Real-Time PCR Detection System (Bio-Rad). PCR conditions were chosen as follows: initial denaturation for 3 minutes at 95°C, 40 cycles of denaturation for 10 seconds at 95°C, annealing for 15 seconds at 55°C and elongation for 30 seconds at 72°C. SYBRgreen fluorescence intensity was determined during the elongation phase. A final denaturation was performed for 30 seconds at 95°C. This was followed by generation of a melting curve, starting from 60°C to 95°C. Temperature was raised in 0.5°C increments, holding each temperature for 7 seconds. The melting curve allowed to distinguish between amplification of PCR products and primer dimers. Only primers that did not lead to the amplification of primer dimers were used. Threshold cycles (CT) were determined by arranging a fixed threshold position at 10000 relative fluorescence units (RFU).

Primer sequences and accession numbers of all investigated genes are listed in annex table A.1 – A.3.

2.5.2 RT-PCR data analysis and statistics

The relative transcript number of target genes was investigated by quantitative or semi-quantitative RT-PCR, respectively, from at least three independent embryo tests. Gene expression data obtained by quantitative RT-PCR was analysed with the *Q-Gene* software (Muller *et al.* 2002) comparing threshold cycle numbers of both target gene and reference gene (*β-actin*) amplification. Threshold cycles of technical triplicates of target and reference gene were averaged. For the calculation of normalised expression, PCR amplification efficiencies (E) of the used primer pairs were taken into account. For the determination of amplification efficiency of a set of primers, the slope of

amplification of different dilutions of cDNA standards was applied in the following equation (1):

$$E = 10^{(-1/slope)} \quad \textbf{(1)}$$

Primer efficiencies were calculated as percentage of the optimal slope of a two-fold amplification per cycle. Efficiencies of all investigated genes are listed in annex table A.1. Based on the respective PCR amplification efficiencies, the ratio of mean threshold values of the target gene to the reference gene was determined to calculate the mean normalised transcript number. The transcript number represents the relative transcription level of the target gene in comparison to the reference gene.

Differential expression obtained by quantitative or semi-quantitative RT-PCR showed similar concentration-dependent trends in each replicated experiment, but the level of expression fluctuated between experiments. Therefore, a data standardisation that is common in multivariate statistics was applied (Afifi & Clark, 1996). Gene expression data of each experiment were divided by the mean that was calculated from all concentrations of the respective experiment. The resulting mean of this standardised data set is equal to one. The advantage of this technique over other approaches - such as the representation of the data as percentage of the expression in controls – is that variation of control data is visible.

Normal distribution was confirmed by the Shapiro-Wilks-W-test ($p < 0.05$) based on the residues of log-transformed data using the software Statistica 7.1 (StatSoft Europe GmbH, Hamburg, Germany). Statistically significant difference of log-transformed data was evaluated with parametric ANOVA and Dunnet`s Test ($p<0.05$) using the software GraphPad InStat 3.01 (GraphPad software, San Diego, California, USA).

2.6 Microarray experiments

2.6.1 Procedure and data aquisition

3,4-DCA concentrations for microarray experiments were selected based on the determination of lethal and effect concentrations (LC/EC), NOEC and LOEC. The

selected concentrations represented ¼ of the LOEC and the LOEC for toxic effects, i.e. 3.1 μM and 12.4 μM, respectively.

Spotted oligonucleotide microarrays with a 14 k zebrafish oligonucleotide set (MWG Biotech AG, Ebersbach, Germany) were produced by the Genome Technology Centre Leiden (University of Leiden, the Netherlands, for details see, (Meijer *et al.* 2005) and were provided by the Department III Genetics of the Max Planck Institute for Developmental Biology in Tuebingen, Germany.

Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was treated with 10 units DNAse I per μg RNA (Roche) for 15 minutes at 25° C and further purified by phenol-chloroform extraction using phase lock tubes (Eppendorf, Hamburg, Germany). Concentration of RNA was measured by absorbance at 260 nm and 280 nm. Integrity of RNA was confirmed by gel electrophoresis.

The hybridisation protocol was kindly provided by the Department III (Genetics) of the MPI for Developmental Biology (Tuebingen) and is based on the publication of Yu *et al.* (2002). A total of 10 μg of DNAse I-treated and purified total RNA were mixed together with 0.38 units (10 μg/μl) $(N)_6$ Random Hexamer primers (Amersham Biosciences, GE Healthcare, Munich, Germany) to a final volume of 18.5 μl and incubated at 70°C for 10 minutes. For indirect labelling of cDNA to the cyanine dyes, a reverse transcriptase labelling mixture including aminoallyl-UTP was added to the RNA to achieve a final concentration of 10 mM dithiothreitol (DDT), 1mM dATP, dCTP, dGTP, 0.6 mM dTTP, 0.4 mM aminoallyl-dUTP (aa-dUTP, Ambion, Huntingdon, UK) and 400 U of SuperScript II RT (Invitrogen) in 30 μl of 5x first strand reaction buffer. This reaction was incubated at 42°C for 14 hours to generate aminoallyl-labelled cDNA.

RNA was hydrolysed by 10 μl of 0.5 M EDTA and 10 μl of 1 M NaOH and incubated at 65°C for 15 minutes. To neutralise the reaction 10 μl of 1 M HCl were added. Unincorporated aa-dUTP and free amines were removed using the QIAquick PCR

purification kit (Qiagen). The samples were dried in a vacuum centrifuge. Aminoallyl-labelled cDNA was resuspended in 4.5 µl 0.1 M sodium carbonate buffer (pH 9.0) and mixed with 4,5 µl of Cy3 or Cy5 monoreactive dye (Amersham Biosciences, Munich, Germany) in DMSO. Dye coupling was performed for one hour at room temperature in the dark. Uncoupled dyes were removed using the QIAquick PCR purification kit (Qiagen). Percentage of labelling was calculated from absorbance at 550 nm for Cy3-labeled reactions (29 – 39 %) and 650 nm for Cy5-labeled reactions (62 – 100 %). The two labelling reactions were combined, vacuum dried and resuspended in 32 µl hybridisation buffer (50% formamide, 5x SSC, 0.1% SDS and 0.1 mg/ml salmon sperm DNA). The samples were denaturised by heating to 95°C for 3 minutes. A volume of 30 µl of the reaction were hybridised to the 14 k zebrafish oligonucleotide slides. The slides were covered by cover slips, inserted into hybridisation chambers and incubated for 14 hours at 42°C in a water bath. The slides were washed in 0.1% SDS, 2x SSC for 10 minutes, followed by three additional washes for 10 minutes in 0.2x SSC, 0.1x SSC and 0.05x SSC at room temperature. Finally, the slides were dried by centrifugation (1000 rpm, 5 minutes). Details on the preparation of media and stock solutions are listed in annex A.6.

Microarray hybridisations were performed for all replicates of samples treated with 3.1 µM (n = 2) and 12.4 µM 3,4-DCA (n = 3). For every single treatment "dye-swaps" were performed, i.e. hybridisation of each experimental sample to the microarray slides was carried out twice to obtain a pair of cyanine 3/5 and cyanine 5/3 labelled cDNAs of control embryos and 3,4-DCA treated embryos.

2.6.2 Microarray data analysis and statistics

The hybridised slides were scanned at 10 µm resolution using the ScanArray Express Scanner (Perkin Elmer, USA) at 532 nm (Cy3) and 635 nm (Cy5). The laser power of the scanner was adjusted between 80% to 100% and the PMT (photo multiplier) value was preset between 60 and 80, depending on the amount of background fluorescence and the saturation of signals.

Microarray data were analysed using the TM4 software package (http://www.tm4.org/, Saeed *et al.*, 2003). TIGR spot finder 3.0.0 beta was used to identify spots on the array and to assess the quality of spots for downstream analysis. The quality assessment for hybridised spots commonly applied using microarray analysis software leads to the rejection of strongly induced or repressed genes that do not feature any hybridisation in either the control or treated sample. These spots are defined of poor quality and are disregarded in the following analysis. Therefore, a minimal threshold value for spots with negative or very low fluorescence intensity was assigned. This minimum threshold value was based on a standard offset intensity of 1000, which was close to the weakest fluorescence of a spot identified by the TIGR software. Strongly induced genes remained in the data set for subsequent normalisation and significance analysis.

TIGR MIDAS 2.19 was then used for data normalisation by the LOWESS (LOcally WEighted leaSt Squares) algorithm (Quackenbush, 2002). In order to remove highly variable, low intensity signals, 30 % quantiles of the spots with the lowest fluorescence intensity were excluded after LOWESS normalisation. In order to keep highly induced or repressed genes, the spots were only excluded when both channels had a low fluorescence. Furthermore, genes for which spots were detected in less than 4 of the 6 hybridisations were also rejected. In case that spots of one or two hybridisations could not be identified, fluorescence data for these spots were calculated by "k-nearest neighbour" imputation (number of neighbours = 10) as part of the significance analysis (see below).

Genes with significant altered expression patterns were identified by a modified t-statistic (SAM = significance analysis of microarrays; Tusher *et al.*, 2001; http://www-stat-class.stanford.edu/~tibs/clickwrap/sam.html). The relative difference *d(i)* in gene expression as a comparison of the ratio of gene expression to the standard deviation in the data for a gene is described by the equation:

$$d(i) = \frac{\bar{x}_T(i) - \bar{x}_C(i)}{s(i) + s_0} \qquad \textbf{(2)}$$

where $\bar{x}_T(i)$ and $\bar{x}_C(i)$ are defined as the average levels of expression for gene (i) in the states *T* (treatment) and *C* (control), respectively. *s(i)* is the standard deviation of

repeated expression measurements and is described as follows:

$$s(i) = \sqrt{a\left\{\sum_m \left[x_m(i) - \bar{x}_T(i)\right]^2 + \sum_n \left[x_n(i) - \bar{x}_C(i)\right]^2\right\}} \qquad \textbf{(3)}$$

where $\sum_m$ and $\sum_n$ are summations of the expression measurements in states *T* and *C*, respectively, $a = (1/n_1 + 1/n_2)/(n_1 + n_2 - 2)$, and n_1 and n_2 are the numbers of measurements in states *T* and *C* (Tusher *et al.*, 2001).

The relative difference *d(i)* can be calculated between treatment and control samples, but also between dye swaps, replicated hybridisations etc. and for a permutation of the data (figure 2.1). The so-called "two-class SAM" is based on permutations of the data set and a user-defined threshold, which determines the number of expected falsely positive regulated genes. "Two-class SAM" can only be applied for single labelled arrays or hybridisations to a reference sample. For double hybridisations of control and treatment sample, as in the microarray approach presented in this thesis, a so-called "one-class SAM" was applied. For the performance of permutations in "one-class SAM" a new data set is generated by multiplying each log 2 transformed expression value at random by either -1 or +1.

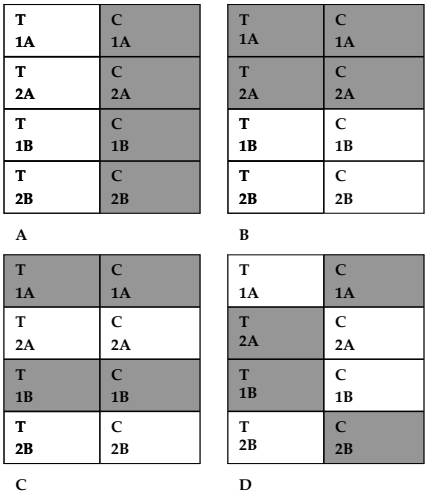

Figure 2.1 Relative difference *d(i)* in gene expression: A = between treatment (T) and control (C), B = of dye swaps (A and B), C = of replicated hybridisations D = of permutations of the data set (modified from Tusher *et al.*, 2001)

The average expression difference of permutations performed by both "two-class" and "one-class SAM" is compared to the observed difference. The difference of observed

values from expected value is quantified by a SAM score (d) for each gene. A high SAM score indicates a high conformity of a gene expression value between replicated hybridisations and/or a strong difference in expression levels between control and treatment samples. The user defined threshold can be adjusted to a certain interval of ratio of expected and observed values (delta). Values beyond this threshold are considered to be significant differentially expressed genes. As delta decreases, the number of identified differentially expressed genes increases, but at the cost of an increasing number of falsely detected regulated genes. Randomly selected genes are indicated by a false discovery rate (FDR). Elevating delta causes a decrease of identified differentially expressed genes, but also a decreased FDR. For analysis of embryos exposed to 3,4-DCA, an appropriate delta was chosen to identify significant genes with the lowest possible FDR.

2.7 Functional manipulation studies

To evaluate the effect of differential gene expression for 3,4-DCA toxicity, overexpression and knock down studies were carried out. Accordingly, mRNA or short interfering RNAs (siRNA), specific for the genes of interest, were injected into one-cell-stage embryos, which were immediately exposed to 3,4-DCA.

2.7.1 Preparation of sense-RNA for overexpression studies

The cDNA full-length clones of *cyp1a, nrf2, maft* and *ho-1* were purchased from the RZPD (German Resource Centre for Genome Research, Berlin, Germany http://www.rzpd.de/; table 2.1). Clones were obtained as inserts in different vectors (pCMV Sport 6.1, Invitrogen and pME 18S-FL, RZPD, see maps in A.7.1, A.7.2), with a gene for ampicillin resistance and were used for transformation of chemical competent E. coli colonies (TOP 10, Invitrogen). Colonies were cultured at 37°C in LB medium containing 50 µg/ml of ampicillin over night. Successful transformation was checked using 2 µl of the culture suspension as template of a colony-PCR. PCR was performed as described above (2.4), using Universal M13 primers (Invitrogen, see annex table A.2) with an initial long denaturation cycle of 5 minutes. 1.5 ml of bacterial culture were

used for plasmid DNA isolation and purification using the Qiaprep Spin Miniprep Kit (Qiagen).

Only the vector pCMV Sport 6.1 exhibited a multiple cloning site flanked by a 5'-SP6 polymerase promoter region and a 3'-polyadenylation sequence (SV 40 polyA). Thus, *in vitro* transcription of inserts at the multi cloning site using the SP6 polymerase was only possible for clones of this vector. Since only *ho-1* was cloned into pCMV sport 6.1, a subcloning of the genes *cyp1a*, *nrf2* and *fzr1* was necessary. Restriction sites of endonucleases for subcloning were identified using the software AnnHyb 4.931 (freely available at http://bioinformatics.org/annhyb/). The full-length clones of *cyp1a*, *nrf2* and *fzr1* were excised from pME 18S-FL using different restriction enzymes (table 2.1). All digestion steps were carried out at 37°C over night, followed by purification with the Qiaquick PCR purification kit (Qiagen). Digested samples were loaded on a 1% agarose gel; inserts were cut out and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen).

For ligation of *cyp1a* and *nrf2* into pCMV Sport 6.1, appropriate restriction sites had to be added to the excised and purified fragments by PCR using Turbo-Pfu polymerase (Stratagene Europe, Amsterdam, The Netherlands) and primers with specific restriction enzyme recognition sequences. Amplification was performed for 30 cycles with an elongation time of 2.5 minutes (for primer information see table 2.1). The target vector pCMV Sport 6.1 was digested with the corresponding restrictions sites to obtain ligation compatible 5'-overhanging ends, purified and finally extracted from a gel. Ligation of *cyp1a*, *nrf2* and *fzr1* to pCMV Sport 6.1 was conducted using T4 Ligase (Fermentas) at 15°C over night. Vectors carrying either *cyp1a*, *nrf2* and *fzr1* or *ho-1*, respectively, were inserted into chemical competent E. coli (TOP 10). Positive clones were identified by colony-PCR with clone specific primers (see annex table A.2). Plasmids were isolated from bacteria and linearised adjacent to the polyadenylsation sequence.

For the synthesis of sense mRNA, the linearised and purified plasmid DNAs were *in vitro* transcribed using the Sp6 mMessage mMachine kit (Ambion). In brief, in the

presence of the SP6 polymerase, mRNAs with a 5'- 7-methylguanosine-cap structure were synthesised. Since the structure of the cap analog of the kit prevents the incorporation at any other position in the RNA molecule, the 7-methylguanosine-cap structure is only linked to the 5'-terminal G of the transcript. DNA templates were removed by digestion with DNAse I and mRNAs were purified using the RNeasy Mini Kit (Qiagen). mRNA yields were determined by photometric absorbance measurement at 260 nm. Sizes of the *in vitro* transcription products were checked by a denaturing agarose gel containing formaldehyde (see annex A.8).

In order to identify potential unspecific effects of mRNA injection, total mRNA was purified from total RNA (table 2.5). Total RNA was derived from 48 hpf zebrafish embryos and isolated as described above. Purification of total mRNA was performed using the Qiagen Oligotex mRNA Mini Kit (Qiagen).

Table 2.1 *In vitro* transcription of the full-length cDNAs *ho 1*, *nrf2*, *cyp1a* and *fzr1*. Clones for *nrf2*, *cyp1a* and *fzr1* were subcloned in the overexpression vector pCMV Sport 6.1 to allow *in vitro* transcription using Sp 6 polymerase. Appropriate restriction sites for the ligation of *cyp1a* and *nrf2 were* added by PCR. Highlighted nucleotides of primers represent the restriction sites of the appropriate enzymes.

full-length clone (complete cds)	ncbi accession number & sequence length	original vector	restriction enzymes for excision		primer (5′- 3′)*	restriction enzymes for ligation	target vector
Heme oxygenase 1 (ho 1; hsp32)	BC061954 (1279 bp)	pCMV-SPORT6.1	-		-	-	-
NF-E2 related factor2 (nrf2)	BC045852 (1761 bp)	pME18S-FL3	Xho1	forward	5′GCGCG***CCGGACCG***ATGATGGAGATTGAAATGTC′3	RE5′: RsrII, RE3′: NotI	pCMV-SPORT6.1
				reverse	5′CGCGCGC***GCGGCCGC***CTAGATATTCTTCACAAGAG′3		
Cytochrome P450 1a (cyp1a)	NM_131879 (2610 bp)	pME18S-FL3	Xho1	forward	5′GCGCG***CCGGACCG***ATGGCTCTGACTATTCTTCC′3	RE5′: RsrII, RE3′: NotI	pCMV-SPORT6.1
				reverse	5′CGCGCGC***GCGGCCGC***CTAGAACCCAGGCTGTGG′3		
Fizzy related protein 1 (fzr1)	BC048038 (2089 bp)	pME18S-FL3	RE5′: EcoRI, RE3′: NotI		-	RE5′: EcoRI, RE3′: NotI	pCMV-SPORT6.1

2.7.2 Preparation of antisense-RNA for knock down studies

For mRNA suppression of each of the zebrafish genes *cyp1a, nrf2, maft* and *ho-1* via RNA interference, three different 21 nt long target mRNA sequences starting with an AA-dinucleotide were identified using the *siRNA target finder* of Ambion (freely accessible at http://www.ambion.com/techlib/misc/siRNA_finder.html). Complete coding sequences of silencing targets were obtained from the ncbi-GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide, table 2.2, page 38). To exclude homology to other genes and to prove gene specificity of siRNAs, the target sequences were compared to the appropriate zebrafish genome database by nucleotide BLAST (BLASTn; Altschul *et al.*, 1990).

DNA antisense and sense template oligonucleotides for siRNAs were designed against the three target sequences of *cyp1a, nrf2, maft* and *ho-1* mRNA (table 2.2, page 38, only those nucleotides, which were finally used for injection are shown). These templates consisted of the 21 nt long sense- or antisense-sequence, respectively, plus a 5` 8 nt long complementary region of the T7 promoter primer (5`-CCTGTCTC-3`). Only sequences of a maximum G/C content of 40% were selected, since higher portions might reduce siRNA activity (http://www.ambion.com/techlib/misc/siRNA_finder.html). The sense template oligonucleotides started with an AA nucleotide at the 5´end, which must not be complementary to the mRNA target sequence, followed by 19 nt sequence complementary to the target sequence (example in figure 2.2). The 5´AA-dinucleotide target sequence provides a 3´terminal UU overhang of the siRNA, which exhibits the highest efficiency of RNA silencing (Elbashir *et al.*, 2001a).

Negative controls – so called mismatch-siRNAs - to every successfully repressed target sequence also were designed. The mm-siRNAs had the same nucleotide composition but with an altered sequence that did not exhibit homology to the target of any other genes (table 2.3, page 39).

Figure 2.2 Example of a siRNA template oligonucleotide (*cyp1a* target sequence no. 10, table 2.2). Underlined sequences indicate the T7 promoter sequence.

Synthesis of siRNA was performed using the *Silencer*® siRNA Construction Kit (Ambion) according to the manufacturers instructions (figure 2.3). Template oligonucleotides were purchased from Invitrogen and hybridised to the linker of the T7 promoter primer. The exo-Klenow DNA polymerase filled the complementary sequences of the T7 promoter primer, as well as of the template oligonucleotides. By adding free nucleotide triphosphates, this double-stranded short DNA sequence could be efficiently transcribed by the T7 polymerase.

Transcription resulted in single-stranded sense- or antisense-siRNA sequences. These sequences were combined to double-stranded siRNA by hybridisation at 37°C over night. Strands of dsRNA were still carrying the 5`terminated T7 promoter sequences, which were removed by single-strand specific RNAse I. In the same reaction, the DNA template was eliminated by DNAse I digestion. The resulting final siRNA was purified from enzymes, nucleotide mixture, salts and oligomers. siRNA concentrations were quantified by photometric absorbance measurement at 260 nm. Every step of the siRNA synthesis was checked by gel electrophoresis using a non-denaturing 12% polyacrylamide gel (PAGE, see annex A.9).

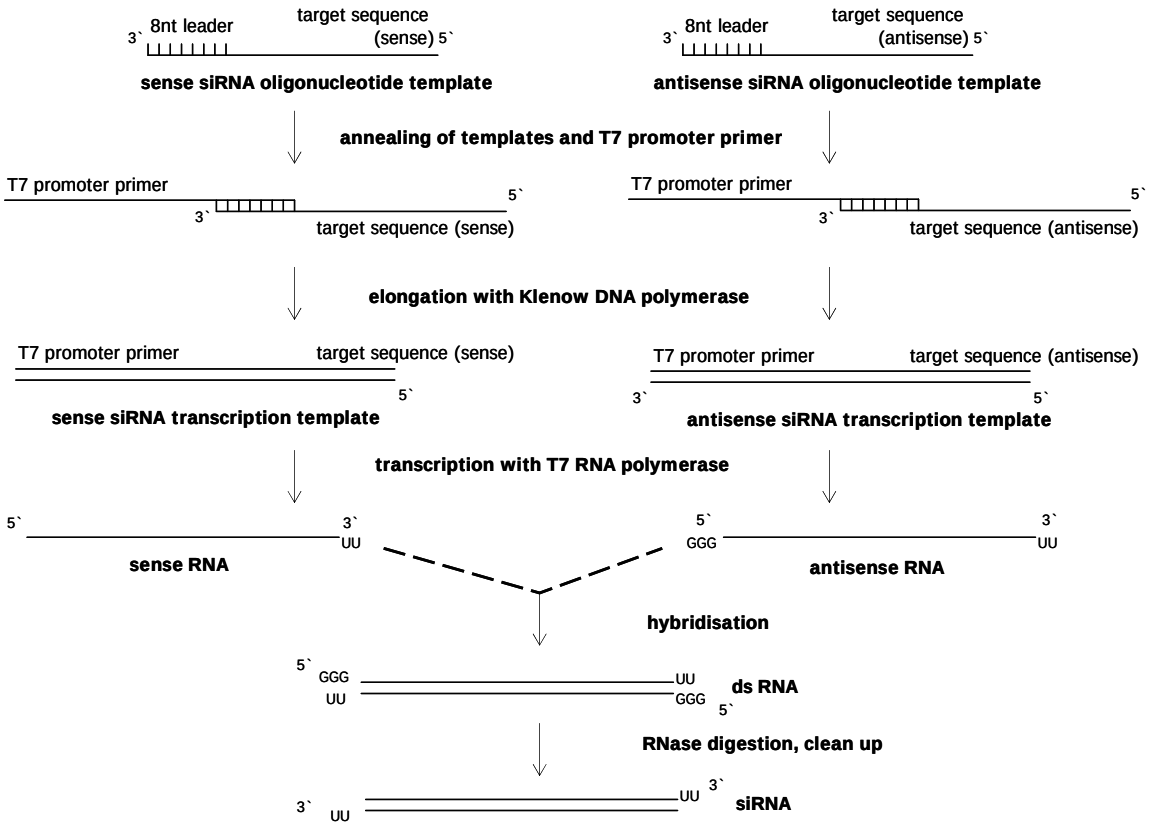

Figure 2.3 Principle of siRNA preparation (modified from Ambion *Silencer*® siRNA Construction Kit manual, 2005)

Table 2.2 Sequence information for injected siRNAs and their corresponding mRNA target sites (selected using the Ambion *siRNA target finder).*

	target sequence					
gene name	**acc. number**	**seq. no.**	**seq. pos.**	**sequence**	**antisense- & sense- siRNA oligonucleotide template**	**siRNA (sense & antisense strand)**
No tail (ntl)	NM_131162	13	232	5′- AATGCAATGTACTCGGTCCTG -3′	5′- AATGCAATGTACTCGGTCCTGCCTGTCTC -3′ 5′- AACAGGACCGAGTACATTGCACCTGTCTC -3′	5`- UGCAAUGUACUCGGUCCUGUU -3′ 3`- UUACGUUACAUGAGCCAGGAC -5′
Cyto-chrome P450 1A (cyp1a)	AY398333	10	176	5′- AAATCGGAAACAACCCACATT -3′	5′- AAATCGGAAACAACCCACATTCCTGTCTC -3′ 5′AAAATGTGGGTTGTTTCCGATCCTGTCTC -3′	5'- AUCGGAAACAACCCACAUUUU -3′ 3'- UUUAGCCUUUGUUGGGUGUAA 5′
NF-E2 related factor2 (nrf2)	AB081314	54	1104	5′- AAGCATGGAGTCAGATTACAA -3′	5′- AAGCATGGAGTCAGATTACAACCTGTCTC -3′ 5′- AATTGTAATCTGACTCCATGCCCTGTCTC -3′	5′- GCAUGGAGUCAGAUUACAAUU -3′ 3′- UUCGUACCUCAGUCUAAUGUU -5′
V-maf musculo-aponeurotic fibrosarco-ma oncogene t (maft)	AB167543	62	1036	5′- AAAGTGCAGATCTGTCGATAA -3′	5′- AAAGTGCAGATCTGTCGATAACCTGTCTC -3′ 5′- AATTATCGACAGATCTGCACTCCTGTCTC -3′	5′- AGUGCAGAUCUGUCGAUAAUU -3′ 3′- UUUCACGUCUAGACAGCUAUU -5′
		66	1087	5′- AATAGGACAAATGTTCCAACA -3′	5′- AATAGGACAAATGTTCCAACACCTGTCTC -3′ 5′- AATGTTGGAACATTTGTCCTACCTGTCTC -3′	5′- UAGGACAAAUGUUCCAACAUU -3′ 3′- UUAUCCUGUUUACAAGGUUGU -5′
Heme oxygenase 1 (ho 1; hsp32)	NM_199678	30	518	5′- AAGATCACACAGAAATCACTG -3′	5′- AAGATCACACAGAAATCACTGCCTGTCTC -3′ 5′- AACAGTGATTTCTGTGTGATCCCTGTCTC -3′	5′- GAUCACACAGAAAUCACUGUU -3′ 3′- UUCUAGUGUGUCUUUAGUGAC -5′

Table 2.3 Sequences of mismatch siRNAs and original- and mismatch-sequence of their corresponding mRNA target sites.

	target sequence					
gene name	**acc. number**	**seq. no.**	**seq. pos.**	**original sequence** **mismatch sequence**	**antisense- & sense- siRNA oligonucleotide template**	**siRNA (sense & antisense strand)**
Cyto-chrome P450 1A (cyp1a)	AY398333	10	176	5′- AAATCGGAAACAACCCACATT -3′ 5′-AAAAACCCGCCCGAAAAATTT-3′	5′- AAAAACCCGCCCGAAAAATTTCCTGTCTC -3′ 5′- AAAAATTTTTCGGGCGGGTTTCCTGTCTC -3′	5′- AAACCCGCCCGAAAAAUUUUU -3′ 3′- UUUUUGGGCGGGCUUUUUAAA -5′
NF-E2 related factor2 (nrf2)	AB081314	54	1104	5′- AAGCATGGAGTCAGATTACAA -3′ 5′-AAATGGACAATCAGTCTAGGA-3′	5′- AAATGGACAATCAGTCTAGGACCTGTCTC -3′ 5′- AATCCTAGACTGATTGTCCATCCTGTCTC -3′	5′- AUGGACAAUCAGUCUAGGAUU -3′ 3′- UUUACCUGUUAGUCAGAUCCU -5′
Heme oxygenase 1 (ho 1; hsp32)	NM_199678	30	518	5′- AAGATCACACAGAAATCACTG -3′ 5′- AAAAAAATTGGCCCTAAGACC-3′	5′- AAAAAAATTGGCCCTAAGACCCCTGTCTC -3′ 5′- AAGGTCTTAGGGCCAATTTTTCCTGTCTC -3′	5′- AAAAAUUGGCCCUAAGACCUU -3′ 3′- UUUUUUUAACCGGGAUUCUGG -5′

2.7.3 Production of one-cell-stage embryos

To avoid a mosaic distribution and mosaic phenotype of embryos injected with sense-mRNA or siRNA, respectively, it is essential to inject only during the one-cell-stage of embryonic development. Different approaches were tested to develop a method to control the fertilisation time and thus, to be able to obtain one-cell-stage embryos at various time points per day.

The following approach was selected for the studies described in this thesis (figure 2.4). Female and male parental fish were kept in separated reservoirs. The day before mating, fish were placed in special tanks with an insert with a grid at its bottom (Aquarienbau Schwarz, Goettingen, Germany). Female and male fish were combined in a ratio of 1:1, 2:1 or 2:2. Males were placed outside, females inside of the inserted tank. Spawning was induced by transferring males into the inserted tank.

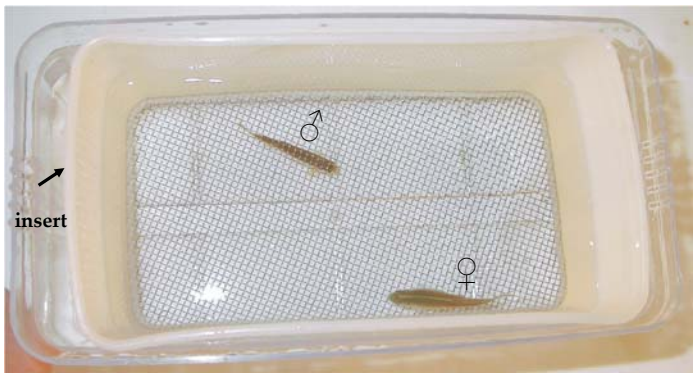

Figure 2.4 A Spawning tank used to obtain embryos of identical stages at defined time points. The male fish is placed outside; the female fish inside of the inserted tank. To induce mating, the male fish is transferred into the insert. Eggs can be collected from the outer tank.

2.7.4 Microinjection and exposure of zebrafish embryos

Injection needles were prepared from borosilicate glass capillaries (type GC100F-10, 1,0 mm OD x 0,58 mm ID, Harvard Apparatus, Edenbridge, UK) using a puller device (Narishige PC-10, Narishige Ltd, London, UK). Capillaries were fixed into a heating element. Pulling was induced by simultaneous heating and drawing of the capillary.

Pulling force was adjusted by weights (47 g). Borosilicate glass needles were filled with the injection solution by means of micro-loader pipette tips (Eppendorf). Fertilised eggs were arranged for injection at the edges of a microscope slide in a petri dish. All residual liquid was removed; the embryos remained attached to the microscope slide. The injection needle was penetrated through the chorion into the cytoplasm of the one-cell-stage embryo. Defined RNA concentrations were introduced by pressure injection of approximately 15 nl using a micromanipulator (Eppendorf Transjector 5246, Eppendorf, Hamburg, Germany) under a stereomicroscope (Leica MZ16F). Pressure of injection was adjusted to gain the appropriate volume with respect to a compensation pressure. Injection time was 0.5 seconds.

The injection volume was determined by measuring the diameter of the drop of liquid injected into paraffin oil using an ocular micrometer (Zeiss, Jena, Germany). The final injected amount was determined by multiplying the concentration of prepared mRNA or siRNA by the injected volume.

Injection of high amounts of mRNA or siRNA, respectively, could result in (non-specific) toxic effects, while injection of low amounts of mRNA or siRNA would not change target gene expression. Thus, volume and concentration of injected RNAs were adjusted to effectively overexpress or knock down gene expression without any toxic effects, which was assessed by checking for developmental disorders in non-exposed embryos. The different injection concentrations of mRNA and siRNA were prepared by dilution in 0.25 M KCl. Finally injected mRNA concentrations were 0.75 – 1.8 ng/nl per embryo, while 3.1 – 9 ng of siRNA were used for repression of transcription (see table 2.4). Injection concentrations of total mRNA or mismatch-siRNAs, respectively, were adjusted to the injected concentration of the corresponding mRNAs or siRNAs by altering the injection volume.

Table 2.4 Injection volume and finally injected concentrations of mRNA and siRNA for overexpression and repression of chemical-sensitive genes in zebrafish embryos.

		concentration (stock) [ng/nl]	[µM]	injection volume [nl]	injected concentration [ng]
mRNA	*cyp1a*	0.07	0.046	15	1.05
	ho 1	0.05	0.038	15	0.75
	nrf 2	0.096	0.058	15	1.35
	fzr 1	0.03	0.016	15	1.8
total mRNA		0.025	-	45	1.13
siRNA	*cyp1a*	0.6	42.9	15	9
	ho 1	0.35	25	15	5.25
	nrf 2	0.64	45.7	15	9.6
	maft (no. 62)	0.21	15	15	3.15
	maft (no. 66)	0.34	24.3	15	5.1
mm-siRNA					
	cyp1a	0.42	30	22.45	9.43
	ho 1	0.58	41.4	10.9	6.32
	nrf 2	0.18	12.9	56.1	10.1

Subsequent to the injection of mRNA or siRNA, one-cell-stage embryos were immediately transferred to glass petri dishes and exposed for 48 hours in a conventional *Dar*T as described in 2.1.

mRNA or siRNA were each co-injected with 0.3% fluorescein isothiocyanate dextran (FITC dextran, Merck) in 0.25 M KCl. FITC dextran injection served as a control for successful injection and in order to identify mosaic embryos. Due to its size, FITC dextran can not leak from the cell and is distributed to the daughter cells with every cell division. To exclude a potential hazardous effect of the co-injected substance alone, embryos were injected exclusively with FITC dextran (n^3=4). No toxic effects, including developmental disorders for control embryos injected with FITC dextran, were observed. Injection of 0.3% FITC dextran also did not alter frequency, nor severity of 3,4-DCA-dependent developmental disorders (99 ± 0.28 % with respect to non-injected embryos; table 2.5).

[3] number of replicated injection experiments using 40 to 60 embryos per experiment

In order to elucidate a gene's function in adaptation or mediation of toxicity, severity and frequency of developmental aberrations in siRNA and mRNA injected embryos as well as in embryos subjected to different control solutions (table 2.5) were observed and documented during embryonic development at 24 and 48 hpf. For documentation of the phenotype, embryos were anesthetised with tricaine methanesulfonate (0.017%; MS 222, Sigma Aldrich) and dechorionated. At the end of exposure, embryos were shock frozen in liquid nitrogen, stored at -80°C and subsequently used for RNA isolation. Repression or overexpression of genes was confirmed by RT-PCR. Expression values were compared to the reference gene cyclophilin A (for primer information see annex table A.1 and A.3).

Table 2.5 Type of injections that were used to study the effect of gene manipulation on toxicity of 3,4-DCA in zebrafish embryos

injection substance	no 3,4-DCA	12.4 µM 3,4-DCA	aim
-	x		negative control
		x	3,4-DCA toxicity without injection
mRNA	x		control for toxic effects of overexpression
		x	effect of overexpression
siRNA	x		control for toxic effects of siRNA
		x	effects of gene silencing
total mRNA	x		toxic effects of mRNA injection
		x	control for specificity of overexpression
mm-siRNA	x		toxic effects of siRNA injection
		x	control for specificity of RNA interference
FITC dextran	x		control for toxic effects of FITC dextran
		x	control for toxic effects of FITC dextran

2.7.5 Data analysis and statistics

For the analysis of altered frequencies of malformations upon functional gene manipulation, only embryos with an even distribution of FITC dextran-fluorescence were selected. Alterations in the frequency of 3,4-DCA developmental aberrations upon exposure in injected embryos were calculated by comparison to the frequency in non-injected embryos:

$$\text{Ratio} = \frac{\text{Percentage of developmental aberrations (injected embryos)}}{\text{Percentage of developmental aberrations (non-injected embryos)}} \quad \textbf{(4)}$$

Statistically significant differences were identified by comparing these alterations in mRNA or siRNA injected embryos to alterations in malformations found in the corresponding control injected embryos (total mRNA or mismatch siRNA). Normal distribution was confirmed by the Shapiro-Wilks-W-test ($p < 0.05$) using the software Statistica 7.1. (StatSoft Europe GmbH). A significance analysis using an unpaired two-tailed t-test ($p< 0.05$) was performed by the software GraphPad Instat 3.01 (GraphPad software).

CHAPTER III

RESULTS

3.1 Toxicity of 3,4-dichloroaniline in *Danio rerio* embryos

Embryos exposed to 3,4-dichloroaniline (3,4-DCA) showed specific developmental disorders such as a reduction of head size and tail length, an incomplete detachment of the tail, an incomplete development of the lens placode, oedemata of different extent (at 24 and 48 hpf) and a reduced pigmentation (at 48 hpf) (figure 3.1). The effect concentrations - based on the summary of all aberrations - were 13.7 µM and 12.4 µM for EC_{50} and LOEC, respectively. The LC_{50} was 1.3 fold higher (17.7 µM) than the EC_{50}.

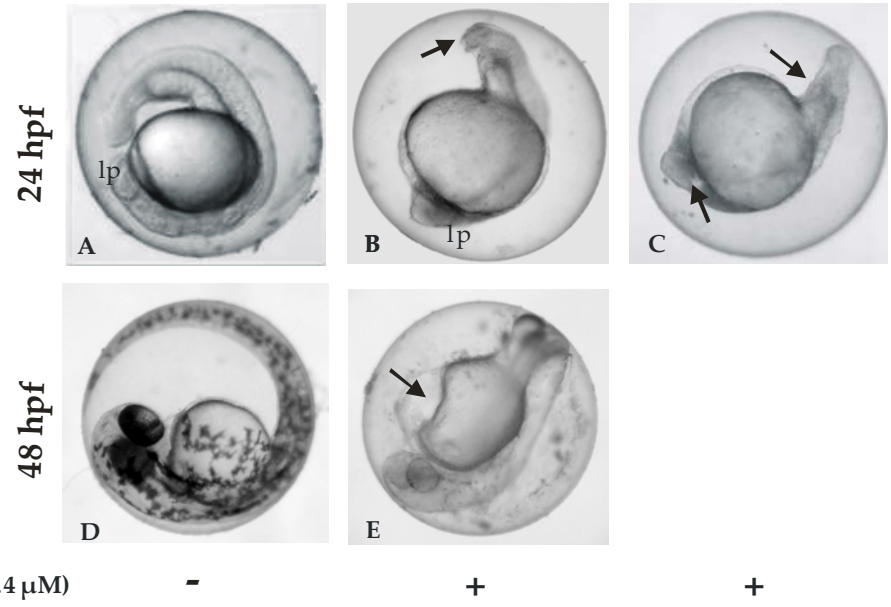

Figure 3.1 Developmental aberrations caused by 3,4-DCA in 24 hpf (B and C) and 48 hpf (E) zebrafish embryos: Malformations are characterised by a reduction of head size and tail length (arrows in B and C), an incomplete or missing formation of lens placodes (C) and an incomplete detachment of the tail at 24 hpf (B and C). At 48 hours of development, oedemata are visible in addition, pigmentation is reduced (E) (A, D = control embryo; lp = lens placode).

Table 3.1 Acute toxicity of 3,4-DCA in zebrafish embryos. Toxicity parameters LC_{50}, EC_{50}, EC_{10}, LC_{10} and NOEC and LOEC were calculated based on nominal concentrations, because measured concentrations were close to the nominal concentrations in most of the treatments.

		3,4-dichloroaniline [µM]	[mg/l]
effect concentrations	LC_{50}	17.7	2.87
	LC_{10}	10.6	1.72
	EC_{50}	13.7	2.22
	EC_{10}	8.1	1.31
	LOEC	12.4	2
	NOEC	6.2	1

3.2 Identification of differentially expressed genes in embryos exposed to 3,4-DCA

3.2.1 Microarray experiments

Microarray technology was deployed to identify differential expression in zebrafish embryos exposed to the polar narcotic substance 3,4-DCA. In order to use sub-lethal and non-toxic concentrations, the LOEC (12.4 µM) and ¼ of the LOEC were selected (table 3.1). Thus, from 2 hpf until 50 hpf, embryos were exposed to 3.1 µM and 12.4 µM 3,4-DCA. Treated und control embryo samples labelled with the cyanine dyes cy3 and cy5 were hybridised to a 14 k oligonucleotide array. After LOWESS normalisation, significant differentially expressed genes were identified by SAM. No significantly altered gene expression was observed in embryos exposed for 48 hours to 3.1 µM 3,4-DCA (figure 3.2 A).

Microarray experiments were repeated using embryos exposed to 12.4 µM 3,4-DCA. SAM revealed 21 genes at a false discovery rate (FDR) of 47 %. At lower FDRs, no significant differentially expressed genes could be detected. In order to remove highly variable, low intensity data, 30 % of genes with the lowest fluorescence intensity were

removed prior to significance analysis. By means of the filtered data set, seven significantly differentially expressed genes at a FDR of 12.8 % were identified (figure 3.2 B, table 3.2). Five genes exhibited an induced expression, while two genes showed a significant repression upon exposure to 12.4 µM 3,4-DCA. The identified genes exhibit different functions in detoxification (*cyp1a*, *ahr2*), cell proliferation (*fzr1*) or basic cell function (*ferritin middle subunit*, *proteasome component c7-i*, *alpha-2-macrogobulin*) (table 3.2).

The average fold-change of differential expression of a gene in replicated exposure experiments identified by microarray ranged from ratios of fluorescence between 1.53 and 6.37 for induced genes and 0.68 and 0.62 for repressed genes. The strongest changes in transcript levels were found for the cytochrome P450 superfamily member *cyp1a* (average fold-change = 6.37). *Cyp1a* mRNA was not detectable or only weakly expressed in control samples. An induction was also observed for the *cyp1a* receptor *ahr2* (average fold-change = 1.68). Furthermore, genes coding for two different ferritins were found to be induced upon 3,4-DCA exposure (average fold-change = 1.94 and 1.53). Both *ferrritins* are distinct genes with only 40% sequence identity and show greatest homology to the human ferritin middle subunit. *Proteasome component c7-I* exhibited a fold-change of 1.55. Genes coding for the fizzy related protein 1 and alpha-2-macroglobulin showed a decrease in expression (average fold-change = 0.68 and 0.62).

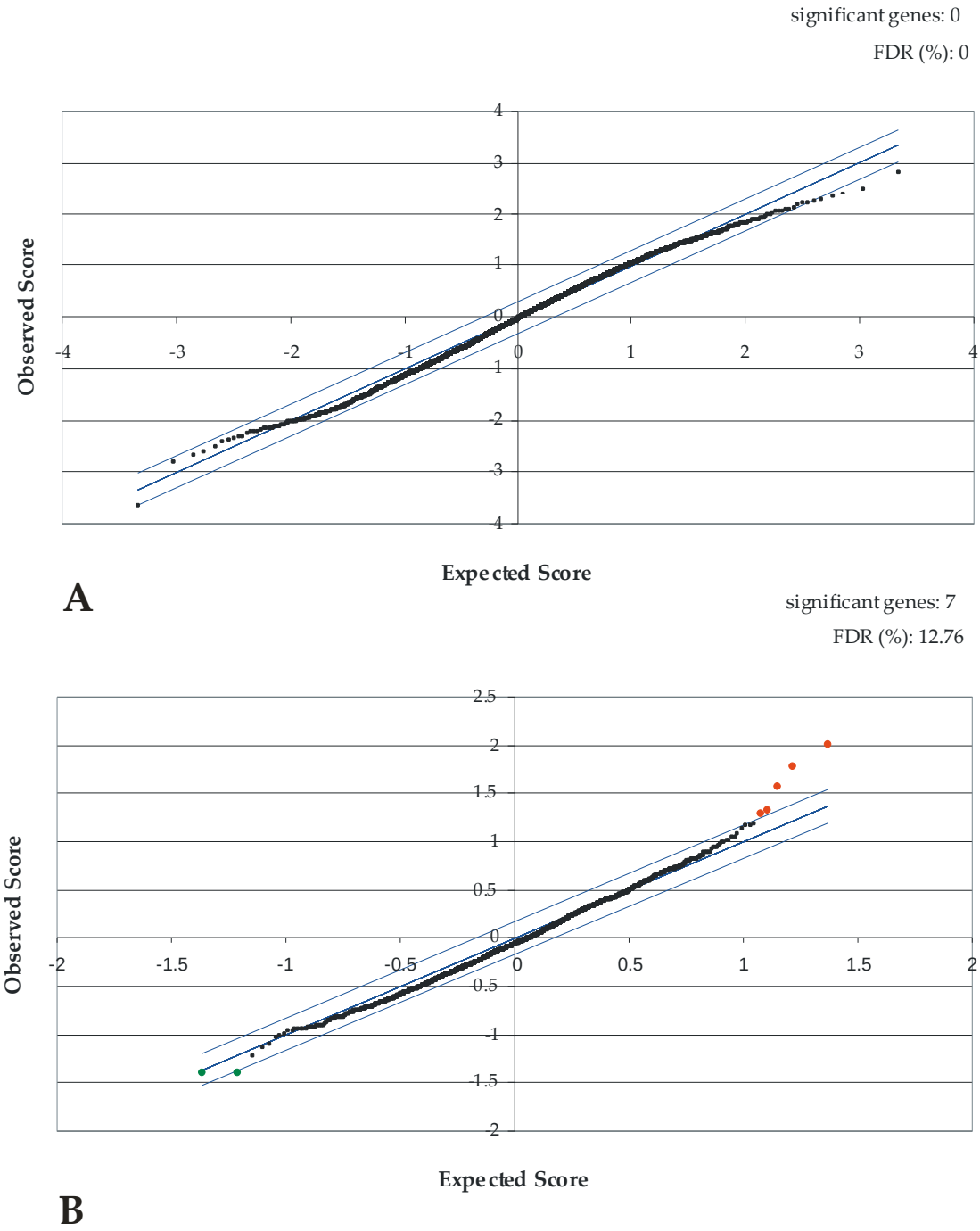

Figure 3.2 Significance analyses of microarrays. **A**: For 3.1 µM 3,4-DCA exposure, no significant differential expression of genes was found by means of SAM. The lower SAM slope does not reveal significant differential expression. Since significant induced genes are characterised by an observed score higher than the expected score, data points had to be above the threshold delta (vice versa for repressed genes). **B**: SAM revealed a significant induction of five genes and repression of two genes in zebrafish embryos exposed to 12.4 µM 3,4-DCA. Significant differentially expression was found below (repression) and above (induction) of a user-defined threshold. Genes close to that threshold exhibit a high likelihood to be false positives (green: repressed genes; red: induced genes).

Table 3.2 Significant differentially expressed genes in 50 hpf embryos exposed for 48 hours to 12.4 µM 3,4-DCA. The table shows expression ratios of individual hybridisations revealed by a 14 k zebrafish oligo array.
* No spot could be identified in this hybridisation.

				ratio treated sample/control sample: 12.4µM 3.4-DCA/K					
				slide1	**slide2**	**slide3**	**slide4**	**slide5**	**slide6**
				cy5/cy3	**cy5/cy3**	**cy5/cy3**	**cy3/cy5**	**cy3/cy5**	**cy3/cy5**
gene name	**acc. no.**	**SAM score [d]**	**fold-change**	**significant induced genes**					
cytochrome P450 1A	AF057713	2.01	6.37	6.24	20.98	94.84	-*	-*	5.13
ferritin, middle subunit	BQ783379	1.78	1.94	1.04	1.50	1.73	3.04	2.84	2.30
aryl hydrocarbon receptor 2 (ahr2)	NM_131264	1.57	1.68	2.19	1.10	_*	2.30	1.53	2.07
proteasome component c7-1	BI710610	1.33	1.55	1.40	1.15	2.20	1.40	1.37	2.32
ferritin, middle subunit	BG892155	1.29	1.53	1.08	1.45	1.56	1.39	1.40	2.70
				significant repressed genes					
fizzy related protein 1	AW173921	-1.39	0.68	0.71	0.79	0.83	0.72	0.80	0.81
alpha-2-macroglobulin	BI326783	-1.39	0.62	-*	0.54	0.57	0.91	0.59	0.38

3.2.2 Confirmation of differentially expressed genes by quantitative RT-PCR

In order to confirm the differential expression of genes that were identified by microarray, transcript levels were analysed by RT-PCR. Genes were examined in three replicate experiments that were performed independent of the experiments for microarray analysis. Zebrafish embryos were exposed to 3.1 µM, 6.2 µM and 12.4 µM 3,4-DCA. In some experiments embryos were also exposed to lower concentrations (0.77 µM, 1.54 µM).

Induction of the genes *cyp1a* (cytochrome P450 1a) and *ahr2* (aryl hydrocarbon receptor 1) as well as the repression of *fzr1* (fizzy related protein 1) was confirmed using conventional RT-PCR. mRNA abundance increased (*cyp1a, ahr2*) or decreased (*fzr1*) in a dose-dependent manner (figure 3.3). Differential expression was not confirmed for the two *ferritin*, the *proteasome component c7-1* and *alpa-2-magroglobulin*.

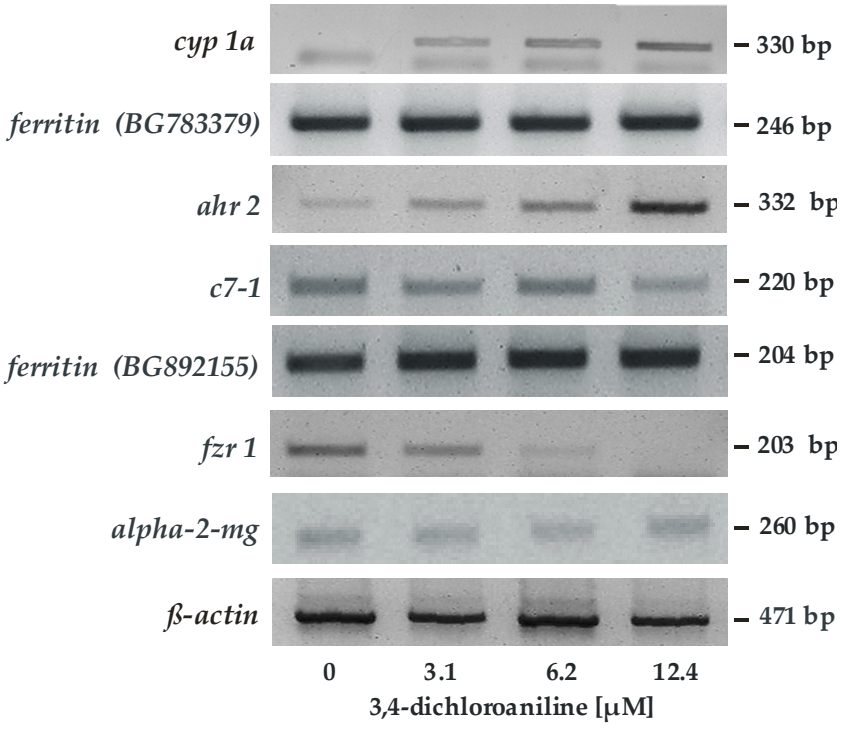

Figure 3.3 RT-PCR of genes that have been shown to be differentially expressed by microarray analysis. Gene expression was analysed in embryos exposed for 48 hours to different concentrations of 3,4-DCA. *ß-actin* and *cyp1a* were amplified for 30 cycles, while amplifications of the remaining genes were performed for 35 cycles. Gel pictures are representative examples selected out of three different embryo tests.

Concentration-dependent differential expression of *cyp1a*, *ahr2* and *fzr1* was also quantified by real-time RT-PCR (qRT-PCR, figure 3.4). All genes were differentially expressed at concentrations below those that elicit toxic effects. The *cyp1a* was found to be significantly induced at 0.78 μM and above (figure 3.4, A). A 9.2 fold elevation of *cyp1a* expression was found upon exposure to 6.2 μM 3,4-DCA if compared to the expression of control embryos. At the highest exposure concentration (12.4 μM) *cyp1a* transcripts were elevated 17 fold. A significant induction of *ahr2* was detected at 6.2 μM 3,4-DCA and 12.4 μM (figure 3.4, B), corresponding to a 2.4 and 2.7 fold induction. *Fzr1* showed a significantly reduced expression at 3.1 μM and above (figure 3.4, C). Transcription of *fzr1* was repressed 2.1 fold upon exposure to 3.1 μM 3,4-DCA compared to the expression in un-exposed control embryos. A 4.2 fold and 5.5 fold repression was observed in embryos exposed to 6.2 μM and 12.4 μM 3,4-DCA, respectively.

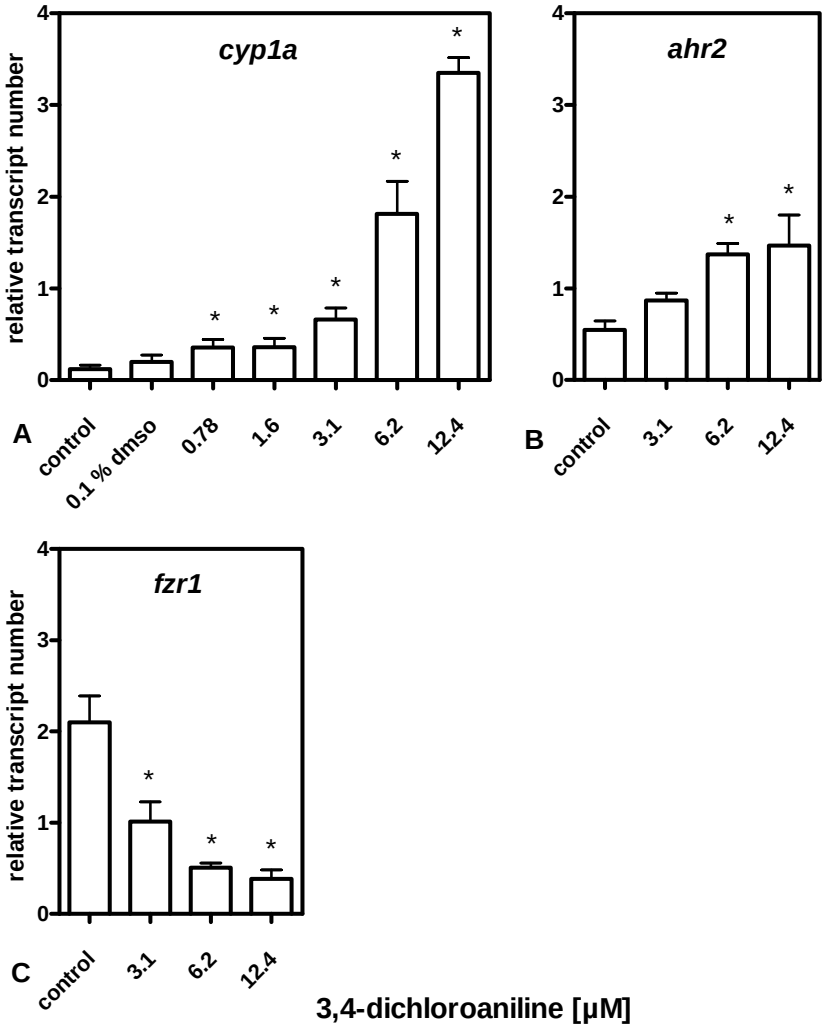

Figure 3.4 Gene expression of *cyp1a* (A), *ahr2* (B) and *fzr1* (C) in zebrafish embryos analysed by quantitative RT-PCR. Embryos were exposed from 2 hours post fertilisation for 48 hours to 3,4-DCA. For normalisation the gene for cytoplasmatic actin was used. Asterisks indicate statistically significant differences compared to control samples based on Dunnet`s test ($p < 0.05$).

3.2.3 Expression analysis of genes not included in the microarray

20 potential candidate genes for differential expression in embryos exposed to 3,4-DCA that were not included in the 14 k microarray were screened by conventional RT-PCR. The selected genes have been described to be differentially expressed in cells or organisms that were exposed to chemicals or they are involved in processes that might be of relevance for the response to xenobiotics. A list of the investigated genes and their functions is given in table 3.3.

Table 3.3 Candidate genes for potential 3,4-DCA-sensitive expression screened by conventional RT-PCR. The selected genes were not among the 14000 genes present on the microarray. Primer sequences are listed in annex table A.1 (√ = differential expression observed by electrophoretic analysis of amplified cDNA fragments).

candidate gene name	accession number	function	reference	differential expression
biotransformation				
transcription factor NF-E2 p45 related factor, nrf2	AB081314	transcription factor of phase2 enzymes and stress proteins	Tagaki *et al.*, 2004	√
transcription factor, mafG1⁺	AB167540	cofactor of transcription factors of phase2 enzymes and stress proteins	Tagaki *et al.*, 2004	√
transcription factor, mafG2⁺	AB167541	cofactor of transcription factors of phase2 enzymes and stress proteins	Tagaki *et al.*, 2004	
transcription factor, mafT	AB167543	cofactor of transcription factors of phase2 enzymes and stress proteins	Tagaki *et al.*, 2004	√
Pi-class glutathione S-transferase 1 (gstp1)	BC083467	phase 2 detoxification enzyme	Suzuki *et al.*, 2005	
Pi-class glutathione S-transferase 2 (gstp2)	AB194128	phase 2 detoxification enzyme	Suzuki *et al.*, 2005	
UDP-glucuronosyl-transferase (ugt)	CD759765	phase 2 detoxification enzyme	Williams *et al.*, 2003	
multixenobiotic resistance				
major vault protein (mvp)	BC049344	part of the large cytosolic ribonucleoprotein particle (vaults); mediates chemical resistance.	Luedeking & Koehler, 2004	
multi drug resistance protein (mrp)	AL591370	ABC transporter; mediate chemical resistance	Luedeking & Koehler, 2004	
(oxidative) stress response				
heme oxygenase (decycling), ho-1 (hsp32)	NM_199678	stress response protein, heme degradation	Alam *et al.*, 2000	√
heat shock protein (hsp 70)	AF006007	molecular chaperone	Lele *et al.*, 1997	√
binding protein (bip)	BC063946	member of the heat shock 70 family; major chaperone of the endoplasmic reticulum (ER)	Ton *et al.*, 2003	
apoptosis				
topoisomerase 2	BQ078151	enzyme, which changes the topology of DNA molecules by cleaving and drilling them	Luedeking & Koehler, 2004	
structure/motality				
myosin light chain 2 (mlc2)	AF081462	important protein for motility and cell structure	Ton *et al.*, 2003	

Table 3.3 (continued)

candidate gene name	accession number	function	reference	differential expression
metabolism				
creatine kinase	BC056706	catalyses the reversible transfer of phosphate from phosphocreatine to ADP; involved in energy storage in skeletal muscles	Ton *et al.*, 2003	
cytochrom-c-oxidase subunit I (cox)	NC_002333	enzyme of the mitochondrial electron transport	Ton *et al.*, 2003	
enolase	NM_200695	catalyses the dehydration of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis	Ton *et al.*, 2003	
glutamate-cysteine ligase (gcl)	BC044532	enzyme of glutathione biosynthesis	Ton *et al.*, 2003	
phosphoglycerate kinase 1 (pk1)	BC046026	catalyses the transfer of phosphate from 1,3-biphosphoenolpyruvate to ADP in glycolysis	Ton *et al.*, 2003	
Ca2+ ATPase	BC051779	ATP depending Ca^{2+} transporter.	Ton *et al.*, 2003	

RT-PCR screening revealed 3,4-DCA-induced expression of the transcriptional activators of phase 2 detoxification enzymes *nrf2* (NF-E2 related factor 2), *maft* and *mafg1* (v-maf musculoaponeurotic fibrosarcoma oncogene homologs), of the stress response protein *ho-1* (heme oxygenase 1) and of the heat shock protein *hsp70* in zebrafish embryos (figure 3.5). For the remaining genes, no alteration in expression was observed (data not shown).

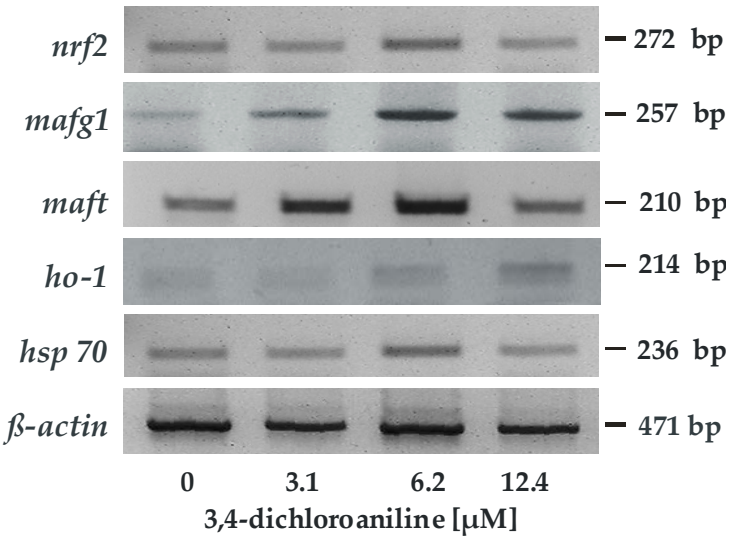

Figure 3.5 RT-PCR of candidate genes for differential expression in response to 3,4-DCA. Gene expression was analysed in embryos exposed for 48 hours to different concentrations of 3,4-DCA. *nrf2, mafg1, maft* and *hsp70* were amplified for 37 cycles, while *ho-1* was amplified for 40 cycles. Amplification of *β-actin* was performed for 30 cycles. Gel pictures are representative examples selected out of three different embryo tests. Only genes with differential expression are shown. For a complete list of genes that were analysed see table 3.3.

Genes with altered expression in response to 3,4-DCA were investigated in detail using quantitative RT-PCR. A statistically significant induction of the genes *nrf2, maft* and *ho-1* was demonstrated at an exposure concentration of 6.2 µM 3,4-DCA (figure 3.6, A, C and D). Transcription of the gene *nrf2* was elevated 2.2 fold compared to the transcription of control embryos. For *maft* and *ho-1* a 6.4 fold and 4 fold, increased transcription was detected, respectively. At the highest exposure concentration tested (12.4 µM), gene expression of *nrf2* and *ho-1* were not statistically significant different from controls. A statistically significant induction of the genes *mafg1* and *hsp70* could not be demonstrated by quantitative RT-PCR (figure 3.6, B and E).

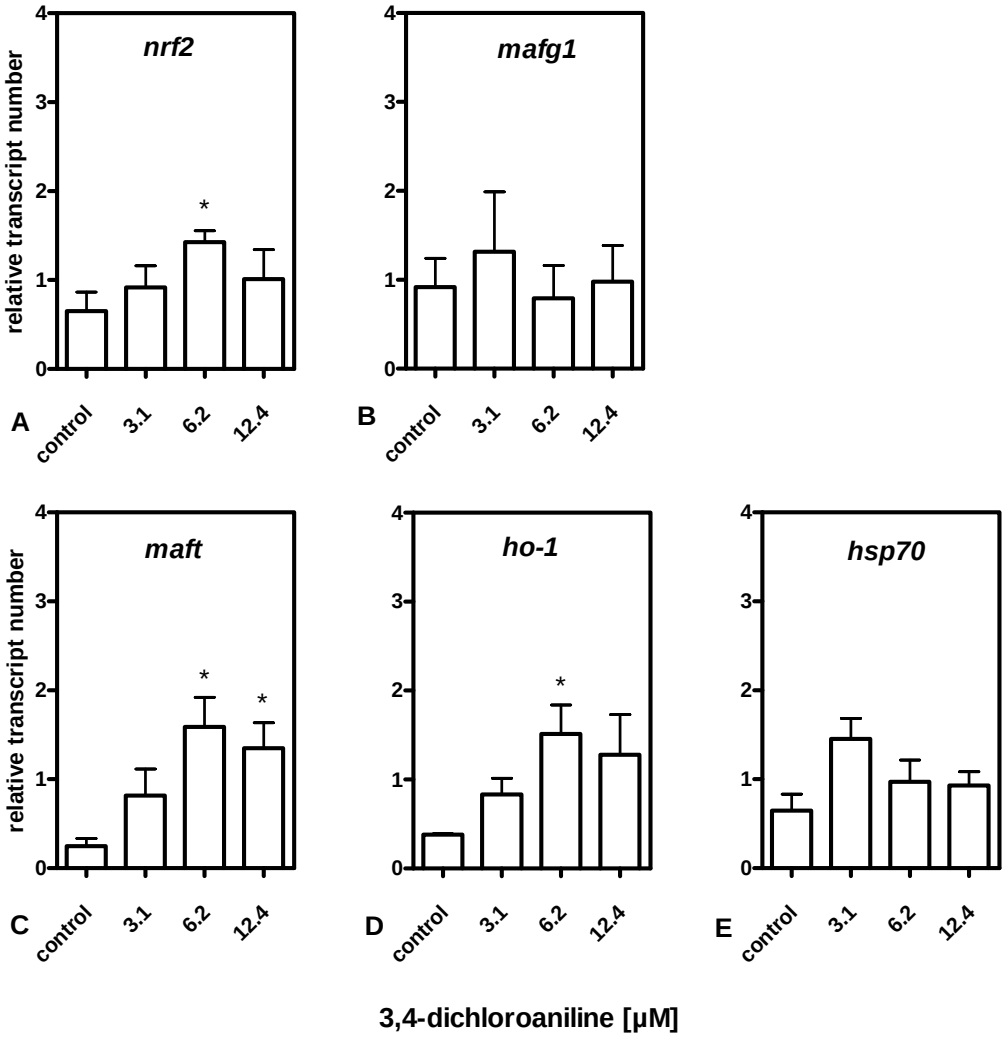

Figure 3.6 Expression of the genes *nrf2* (A), *mafg1 (B), maft* (C), *ho-1* (D) and *hsp70* (E) in zebrafish embryos analysed by quantitative RT-PCR (n=3). Embryos were exposed from 2 hours post fertilisation for 48 hours to 3,4-DCA. For normalisation the gene for cytoplasmatic actin was used. Asterisks indicate statistically significant difference compared to control samples based on Dunnet`s test ($p < 0.05$).

3.3 Gene expression in zebrafish early larvae and juvenile fish exposed to 3,4-DCA

In order to analyse the effect of prolonged exposure and stage-specific sensitivity for gene expression, an early life stage test (ELST) with 3,4-DCA was performed (test was conducted by Dr. M. Tillmann, TU Dresden, Germany).

LOECs, NOECs, EC_{10} and EC_{50} values were calculated based on the occurrence of chorda deformation, oedemata and reduction of pigmentation. The EC_{10} was the lowest at 30 days of exposure (0.27 µM). NOEC and LOEC after 30 days of exposure were determined as 0.11 µM and 0.22 µM, respectively. A mortality of 100 % was observed in samples exposed to the highest tested concentration of 3,4-DCA (1.86 µM). Toxicity data are summarised in table 3.4.

Table 3.4 Chronic toxicity data of 3,4-DCA of an early life stage test (ELST) using zebrafish (data were provided by Dr. M. Tillmann, TU Dresden). Fish were exposed from 0.11 to 1.86 µM 3,4-DCA starting from fertilisation for 30 days. Survival and developmental disorders were analysed at 14 and 30 dpf.

		day 14	day 30
	3,4-DCA	[µM]	
effect concentrations	LC_{50}	1.67	1.11
	LC_{10}	0.48	0.68
	EC_{50}	1.17	1.51
	EC_{10}	0.57	0.27
	LOEC	0.46	0.22
	NOEC	0.22	0.11

Gene expression was analysed in 5 dpf larvae and 30 dpf juvenile fish exposed to concentrations of 3,4-DCA below the LC50. The genes that were analysed, comprised those that were confirmed by quantitative RT-PCR to be differentially expressed in embryos exposed to 3,4-DCA.

Differential gene expression in early larvae and juvenile fish exposed to the ELST with 3,4-DCA was found in the concentration range of LOEC and NOEC concentrations (compare figure 3.7 and table 3.3). With the exception of *fzr1*, all analysed genes showed a differential expression in early larvae (5 day-old fish) in response to 3,4-DCA (figure 3.7 A). An increase of mRNA abundance of these genes was found below exposure concentrations that elicited significant changes in gene expression in 48 hours old embryos.

Ahr2 transcript levels were enhanced upon exposure to 0.22 µM 3,4-DCA and above. The *nrf2* gene induction was found at 0.46 µM 3,4-DCA and above. Transcripts of the genes *cyp1a, maft, mafg1* and *ho-1* in zebrafish early larvae showed an increased abundance at 0.93 µM 3,4-DCA.

In 30 day-old juvenile fish, differential gene expression was analysed only at 0.46 µM and 0.93 µM 3,4-DCA. Induction of the genes c*yp1a, nrf2, maft* and *ho-1* was observed at exposure concentration of 0.46 µM and above. Gene expression of *ahr2* was enhanced at 0.93 µM 3,4-DCA. In contrast to 5 day-old fish, a weak repression of *fzr1* was found in juvenile fish at 0.93 µM (figure 3.7 B).

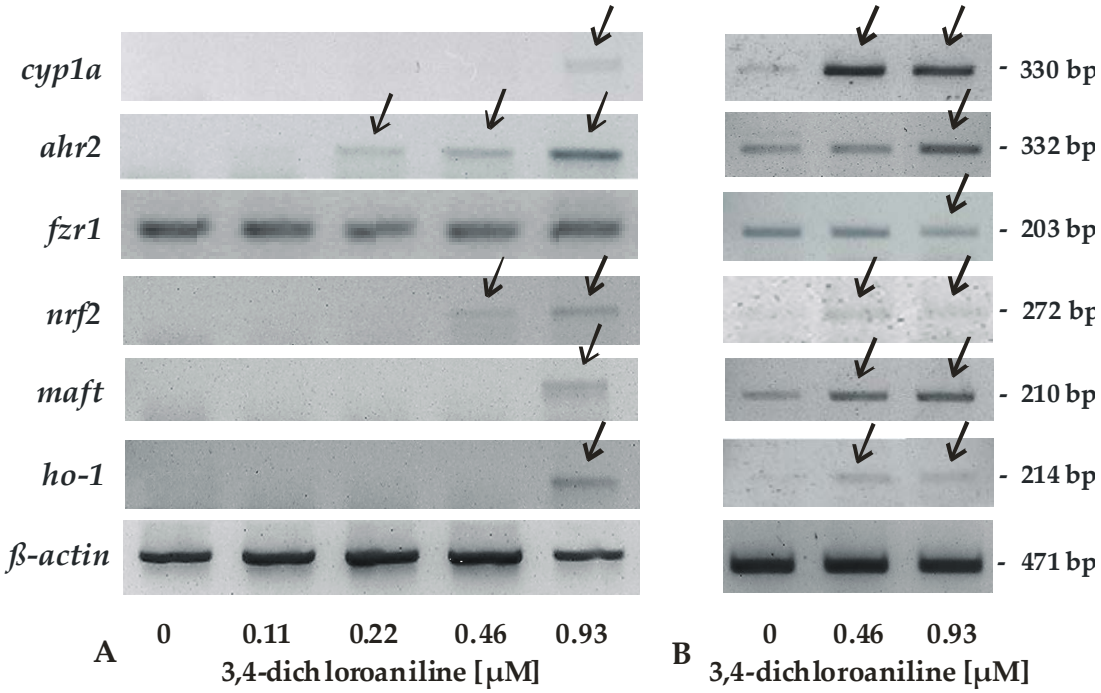

Figure 3.7 Gene expression of *cyp1a*, *ahr2*, *fzr1*, *nrf2*, *maft* and *ho-1* in sac fry stages (5 days post fertilisation, A) and juvenile stages (30 days post fertilisation, B) of zebrafish exposed to 3,4-DCA in an ELST. Zebrafish were exposed from 2 hours post fertilisation for 5 days or 30 days, respectively. Gene expression was analysed by RT-PCR. Transcripts were amplified for 35 cycles. Amplification of *β-actin* (30 cycles) served as constitutive expression. Arrows indicate altered transcript levels in response to 3,4-DCA exposure.

3.4 Effect of β-naphthoflavone exposure on gene expression

Gene expression patterns of 3,4-DCA exposed embryos indicated an Ah-receptor mediated regulation (see chapter IV "Discussion" for detailed consideration). Therefore, embryos were exposed to the Ah-receptor model ligand β-naphthoflavone (BNF). Exposure to BNF should indicate whether or not a model AhR-ligand affects the same genes as 3,4-DCA.

BNF exposure concentrations were adopted from Dong *et al.*, 2002, who found first signs of BNF-toxicity in 50 hpf embryos at 1 μM BNF. Thus, embryos were exposed to 1 μM BNF and below (0.1 and 0.01 μM BNF; n=3). Using semiquantitative RT-PCR (densitometric analysis of gel bands from RT-PCR) the expression levels of the genes *nrf2*, *ho-1*, *cyp1a* and *fzr1* were analysed. In 48 hours old zebrafish embryos, the expression of *cyp1a*, *nrf2* and *ho-1* were induced by BNF in a concentration-dependent manner. A statistically significant induction was observed at 0.01 μM BNF and above for *cyp1a* and *ho-1* and at 1 μM BNF for *nrf2*, respectively (figure 3.8). However, no alterations in *fzr1* transcript abundance were detected. The fold-change induction was not calculated because the densitometric analysis does not allow a linear correlation to transcript numbers.

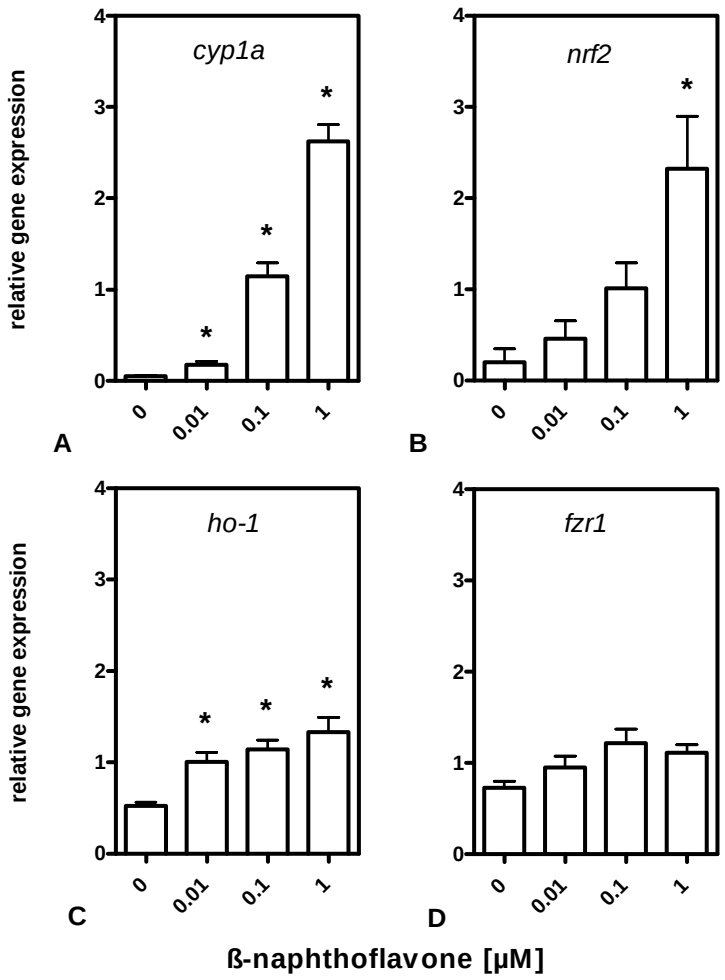

Figure 3.8 Gene expression of *cyp1a* (A), *nrf2* (B), *ho1* (C) and *fzr1* (D) in zebrafish embryos exposed from 2 hours post fertilisation for 48 hours to β–naphthoflavone. Gene expression was analysed by RT-PCR, using 30 cycles of amplification for *cyp1a* and 35 cycles of amplification for n*rf2, ho-1* and *fzr1*, (n=3). Relative gene expression was calculated based on a densitometric analysis of gel bands. *Cyclophilin A* (30 cycles) was used for normalisation of target genes. Primer information for the corresponding genes are listed in annex table A.1. Asterisks indicate statistically significant difference compared to control samples as indicated by Dunnet`s test ($p < 0.05$).

3.5 Characterisation of the relevance of gene expression for 3,4-DCA toxicity

Functional manipulations of toxicant-sensitive genes in zebrafish embryos were performed in order to estimate the relevance of differential gene expression for either mediation of toxicity or adaptation to toxicity. Therefore, gain- and loss of function studies have been conducted by injection of mRNA or siRNA into one-cell-stage

embryos. The genes *cyp1a, ho-1, nrf2, fzr1* (only for mRNA) and *maft* (only for siRNA) were chosen for the examination of gene function. The genes were selected based on their differential expression in 3,4-DCA-exposed embryos and the availability of full-length clones. The *ahr2* was not selected for functional analysis since it is directly linked to the regulation of *cyp1a* and was expected to reveal similar results.

3.5.1 mRNA overexpression

mRNAs of *cyp1a, ho-1, nrf2* and *fzr1* were synthesised by *in vitro* transcription and synthesis was analysed by a denaturing agarose gel electrophoresis (figure 3.9).

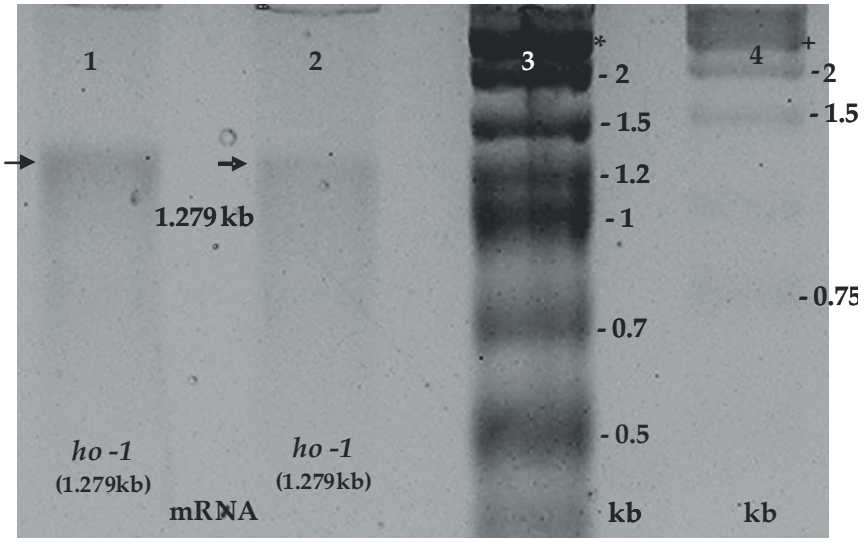

Figure 3.9 Denaturing agarose gel electrophoresis of a synthesised mRNA produced by *in vitro* transcription of an expression plasmid. Only the *in vitro* transcription of *ho-1* mRNA is shown as an example; the length of the *ho-1* mRNA sequence of 1279 bases was confirmed (lane 1 and 2; lane 3 and 4: DNA ladders including fragments of different size, * 100 bp DNA Ladder (lane 3), + 1 kb DNA Ladder (lane 4)). Similar results were obtained for *cyp1a, nrf2* and *fzr1* mRNA *in vitro* transcriptions.

The mRNAs of the selected zebrafish genes for overexpression were co-injected with 0.3% fluorescein isothiocyanate dextran (FITC dextran) into one-cell-stages. The amount of injected mRNA was adjusted to avoid unspecific toxic effects by overexpression of mRNA. Therefore, a dilution series of mRNA was used to specify the amounts that did not induce any malformation in control embryos. Concentrations as high as possible, but below those that elicit non-specific toxic effects, were used in

subsequent experiments.

Toxic side effects due to overexpression included failure of tail formation, deformation of the chorda, distortion of the yolk sac, shrunken head and the occurrence of oedemata (figure 3.10). Based on these experiments total amounts of injected mRNAs of 0.75 - 1.8 ng in an injection volume of 15 nl were selected (table 2.4).

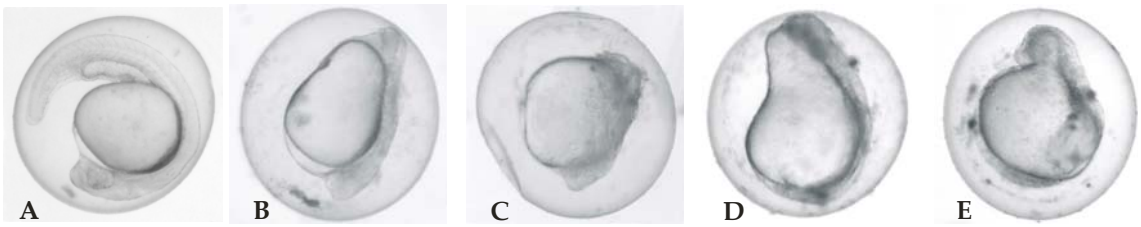

Figure 3.10 Developmental disorders induced by overexpression: Injection of 1.5 and 1.13 ng *ho-1* mRNA (B,C), as well as injection of 5.4 ng and 2.7 ng *nrf2* mRNA (D,E) caused malformations in 24 hpf un-exposed embryos (A).

Upon injection, embryos were immediately exposed to 12.4 µM 3,4-DCA and the degree of toxic effects was quantified by the frequency of developmental disorders (malformations of head, tail and lens placode) at 24 and 48 hpf. RT-PCR was used to confirm the elevation of mRNA abundance caused by mRNA injection (figure 3.11 A). Increase of transcripts of *cyp1a, nrf2* and *ho-1* were found in non-exposed embryos, but were difficult to detect in 3,4-DCA exposed embryos, since 3,4-DCA already induced the expression of these genes. No enhancement of *fzr1* mRNA levels could be detected.

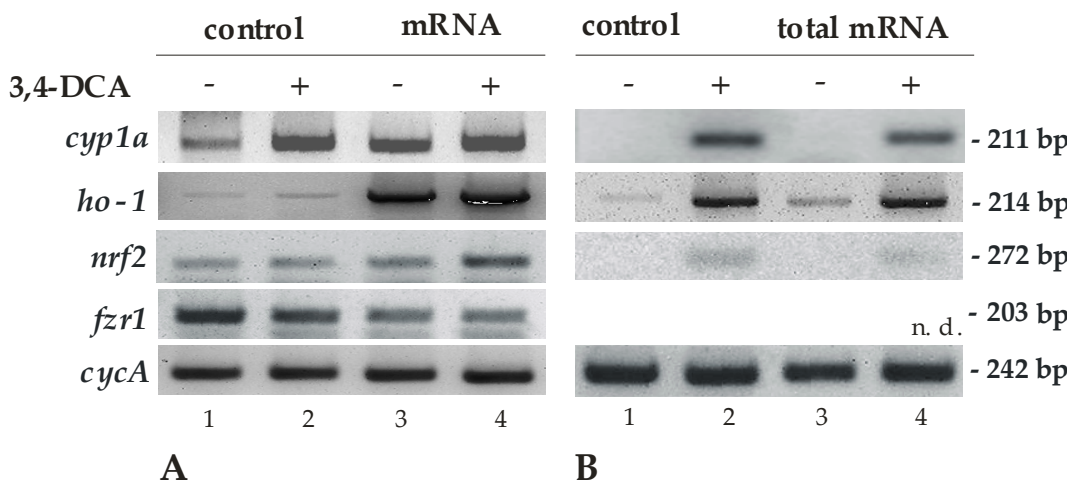

Figure 3.11 Impact of mRNA injection on transcript abundance. **A:** Overexpression of genes in 48 hpf zebrafish embryos following an injection of specific mRNA. By RT-PCR an increase of *cyp1a* and *ho-1* mRNA abundance was observed in non-exposed as well as in 3,4-DCA exposed embryos (lane 3 and 4). Due to the already elevated transcript levels in exposed embryos, only a slight elevation of mRNA levels was observed following mRNA injections. *nrf2* overexpression could only be detected in 3,4-DCA exposed embryos. No alterations of transcripts levels were apparent for *fzr1*-injected embryos. **B:** Injection of total mRNA as a negative control did not alter the transcript abundance of the genes *cyp1a, ho-1* and *nrf2* (n.d. = not determined; lane 3 and 4) in 48 hpf zebrafish embryos (cycles of amplification: *cycA* and *cyp1a* 30 cycles; *ho-1, nrf2* and *fzr1* 35 cycles).

Control injections with total mRNA were performed to assure gene specific effects (table 2.5). mRNA of the target genes is represented in only low amounts in total mRNA samples, thus, no specific effects on the transcript levels can be anticipated. No changes in the transcript abundance of the genes c*yp1a, ho-1* and *nrf2* were detected, as revealed by RT-PCR (figure 3.11 B). Injection concentrations of total mRNA were adjusted to those used in the overexpression studies. Total mRNA control injections did not cause any changes in the frequency of 3,4-DCA dependent developmental disorders (96 ± 17 %; figure 3.12 A – C, left bars).

In embryos that were injected with *cyp1a*, *ho-1* and *nrf2* mRNA, the severity of developmental disorders upon exposure to 3,4-DCA was similar to those found in uninjected control embryos, but the frequency was reduced to 63 ± 24 % (*cyp1a*) and 67 ± 23 % (*ho-1*) and 60 ± 3 % (*nrf2)*, respectively (figure 3.12, A – C, right bars). Embryos injected with *fzr1* mRNA (n=4) did not show any alterations in the frequency of

malformations.

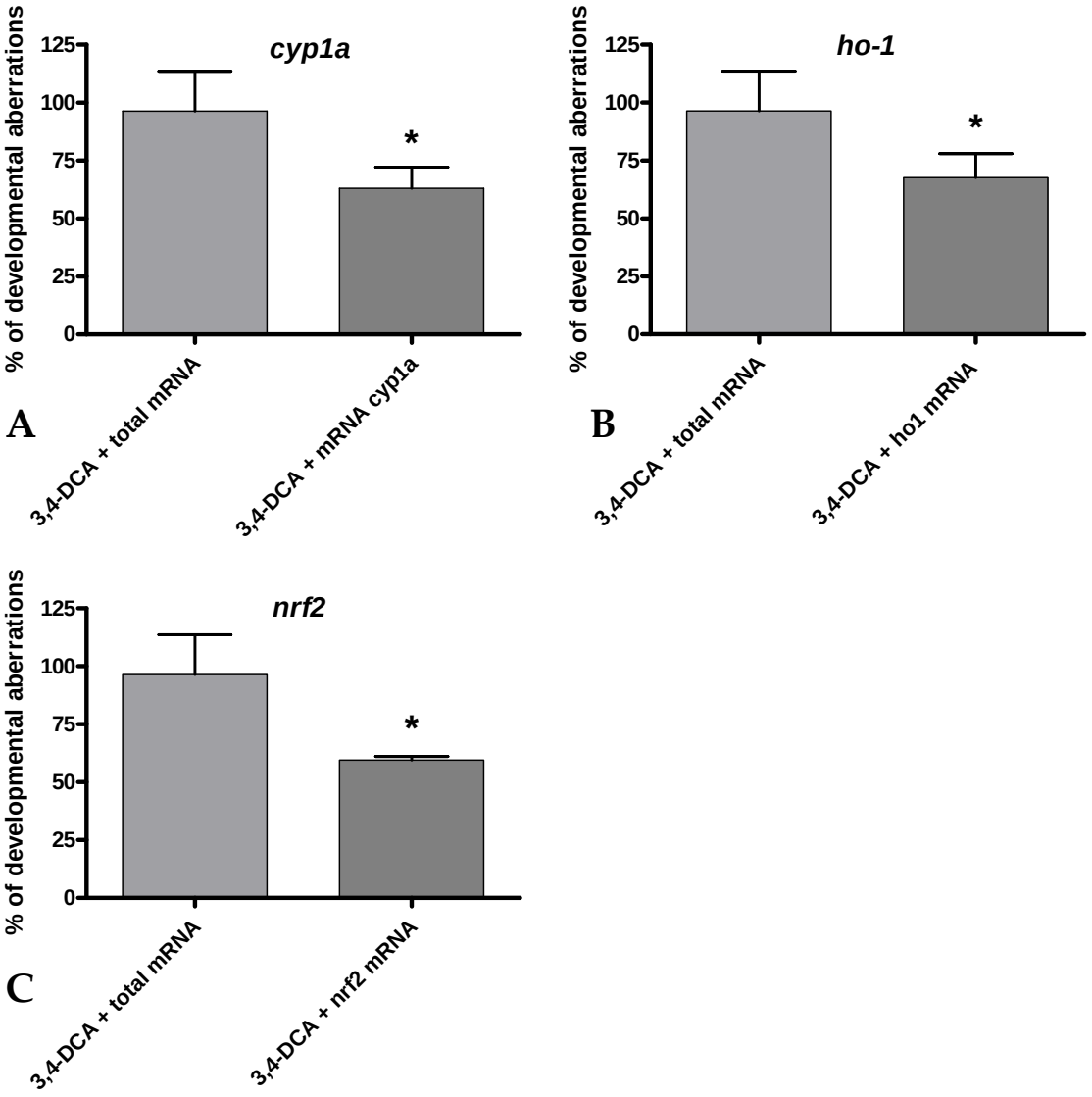

Figure 3.12 Overexpression of the genes *cyp1a* (A, n=7), *ho-1*(B, n=5) and *nrf2* (C, n=3) in embryos exposed to 12.4 μM 3,4-DCA caused a significant reduction of developmental disorders (right bars). Total mRNA (n=6) control injections did not influence the frequency of 3,4-DCA-malformations (left bars). The frequency of developmental disorders was calculated by comparison to non-injected embryos exposed to 3,4-DCA (= 100 %) (*significantly different, if compared to embryos injected with total embryonic mRNA in an unpaired, two-tailed t-test, $p < 0.05$).

3.5.2 mRNA silencing

Establishment of the siRNA-technique

While transient gene knock down using siRNA-technique in cell culture is well established, the application in fish embryos is only rarely used. Therefore, the study of Liu *et al.* (2005) has been used as reference to establish this technique. This study is focussed on injections of siRNA against the gene *no tail* (*Danio rerio ntl*), a factor of mesoderm determination. Upon injection of 4 ng si-*ntl*, Liu *et al.* (2005) reported a frequency of 49 % of *ntl* specific phenotypes. The phenotypes were similar to a knock out mutation, which is characterised by a prominent distortion of the posterior tail (Amacher *et al.*, 2002).

In order to confirm the si-*ntl* specific phenotypes, 5 ng of si-*ntl* were injected into one-cell-stage embryos. siRNA was co-injected with FITC dextran to identify successfully injected embryos. The *ntl* mRNA levels were examined by RT-PCR (figure 3.13).

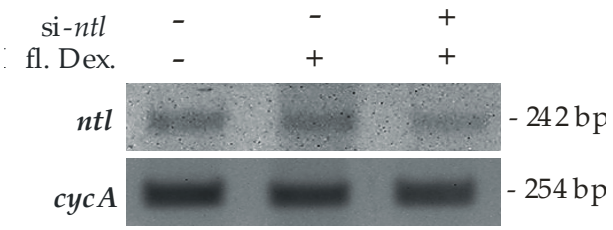

Figure 3.13 siRNA-mediated repression of *ntl*: A slight decrease in transcript abundance of *ntl* could be detected by RT-PCR in 48 hpf embryos injected with 5 ng *si-ntl* (lane 3).

Embryonic development was inspected for the appearance of the *ntl*-phenotype after 24 and 48 hpf. The prominent malformations of the *ntl* mutant phenotype were confirmed: 43 or 50% of FITC dextran-fluorescing embryos showed a total or partial *no tail* phenotype as described by Liu *et al.* (figure 3.14).

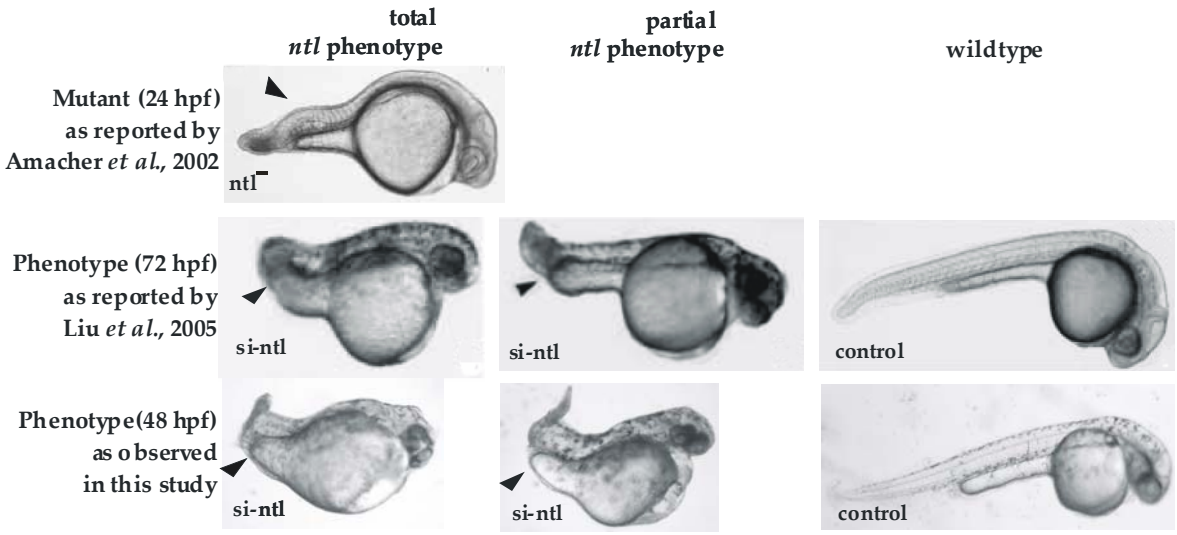

Figure 3.14 Comparison of *no tail* mutant and siRNA-generated phenotypes in zebrafish obtained by Amacher *et al.* (2002), Liu *et al.* (2005) and in this study. Repression of the gene *no tail* is characterised by a posterior deformation of the tail. Embryos were dechorionated for better visualisation of phenotypes.

mRNA repression by RNAi

To study gene function by the repression of transcripts via RNA interference, the 3,4-DCA-sensitive genes *cyp1a, ho-1, maft* and *nrf2* were selected. Since 3,4-DCA is already repressing the expression of the *fzr1*, no siRNA injections were performed for this gene. *In vitro* transcription into siRNA by T7 polymerase was checked by non-denaturing polyacrylamide gel electrophoresis (PAGE, figure 3.15, only *cyp1a* is shown as example).

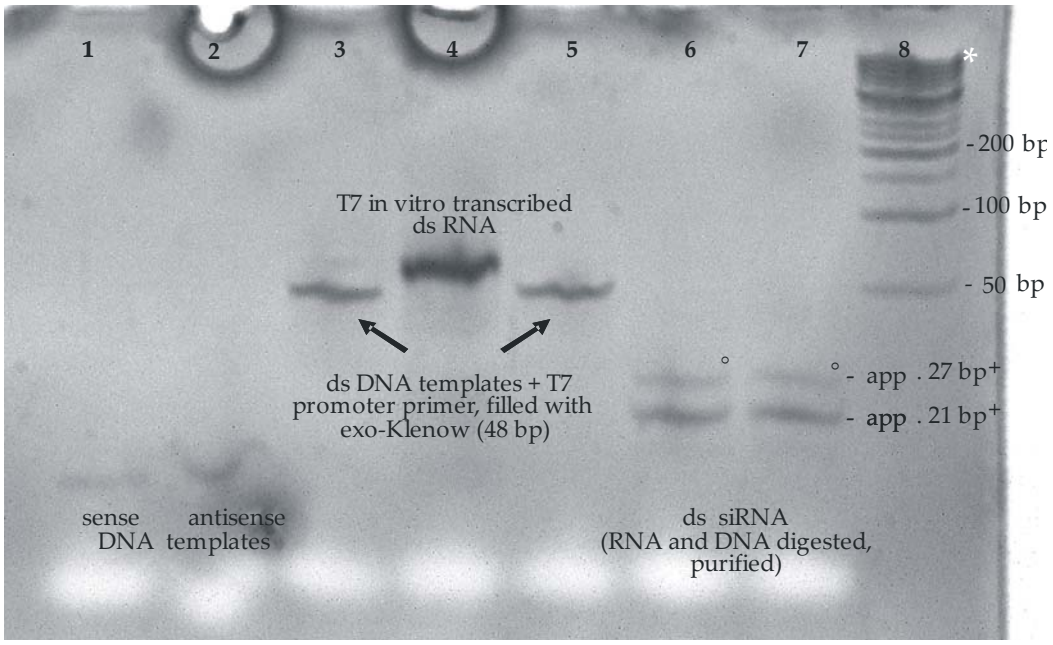

Figure 3.15 Non-denaturing PAGE of si-*cyp1a* synthesis (similar results were obtained for siRNA synthesis of *ho-1, maft* and *nrf2*). Sense- and antisense-oligo DNA templates of *cyp1a* (lane 1 and 2) were hybridised to a T7 promoter primer and gaps of the hybridisation products were filled by an exo-Klenow fragment (lane 3 and 5). *In vitro* transcribed, single-stranded RNA sequences were hybridised (lane 4). Digestion of DNA and of 5`overhanging leader sequences of the double-stranded RNA resulted in siRNA of approximately 21 bp length (lane 6 and 7) (° replicates of *cyp1a* siRNA; * 50 bp DNA Ladder; + final siRNA consisted of 21-22 bp, minor band of larger size corresponds to an incompletely digested 27 bp strand).

Initially, injections of all siRNAs were performed using maximum concentrations, depending on the yield of the siRNA synthesis. According to the selection of appropriate mRNA concentrations for overexpression, the amounts of injected siRNAs were adjusted to avoid toxic effects. The repression of mRNA levels in injected embryos was checked by RT-PCR using primers 5´upstream of the siRNA target sequences. Repression of *cyp1a, nrf2* and *ho-1* transcripts was found in non-exposed embryos and in 3,4-DCA- exposed embryos. No silencing of *maft* mRNA levels could be detected (figure 3.16 A).

Mismatch-siRNAs against *cyp1a, ho1* and *nrf2* were injected as controls for the specificity of the RNA silencing and to assure that alteration in the frequency of malformations are related to specific gene knock down. Injection concentrations were adjusted to those of the corresponding siRNAs (table 2.4). No changes in the transcripts

of genes could be detected in mm-siRNA-injected embryo samples using RT-PCR (figure 3.16 B). Injections of mismatch-siRNAs had no influence on the frequency of 3,4-DCA dependent disorders (mm-si-*cyp1a*: 97 ± 9.7%; mm-si-*ho-1*: 96 ± 6.6%; mm-si-*nrf2*: 102 ± 6.1 %; figure 3.18, left bars).

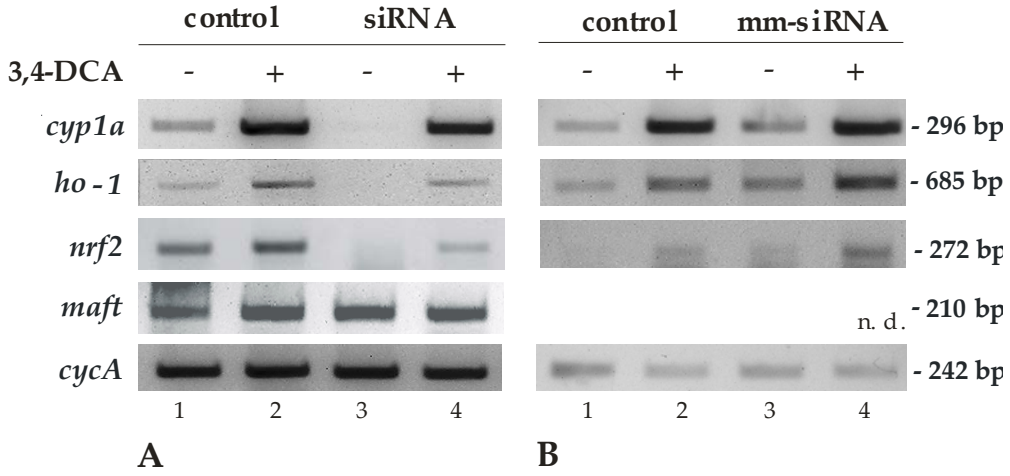

Figure 3.16 Impact of siRNA injections on transcript abundance. **A:** siRNA-mediated mRNA silencing in zebrafish embryos. RT-PCR demonstrated a knock down of transcript abundance of the genes *cyp1a*, *ho-1* and *nrf2* in both 3, 4-DCA-exposed and control embryos (48 hpf). Repression of transcripts is clearly visible in control embryos (lane 3) and less prominent in exposed embryos (lane 4). No repression of *maft* mRNA could be detected upon RNA silencing. **B:** Injection of specific mismatch-siRNA did not repress the transcript levels of the genes *cyp1a, ho 1* and *nrf2* (lane 3 and 4) (n.d. = not determined; cycles of amplification: *cycA* and *cyp1a* 30 cycles; *ho-1, nrf2* and *maft* 35 cycles).

Changes in the severity and frequency of 3,4-DCA-induced malformations upon siRNA and mm-siRNA injection were examined after 24 hpf and 48 hpf. Developmental aberrations in 3,4-DCA-exposed embryos that were injected with si-*cyp1a* or *ho-1* were elevated both in frequency and severity. Some embryos showed a complete lack of the tail, a small head and no lens placode (figure 3.17, D). Frequencies of malformations were elevated by 171 ± 14 % (*cyp1a*) and by 159 ± 38 % (*ho-1*) upon si-RNA injection (figure 3.18, right bars).

A higher sensitivity of embryos towards 3,4-DCA was also found upon injection of si-*nrf2*. Frequency, but not severity of developmental aberrations was enhanced in two experiments by 126 % and 400 % (263% +/- 127%). Further replicates are needed to

confirm these results. Injection of si-*cyp1a*, si-*ho-1* (figure 3.17, C) or si-*nrf2*, respectively, in control embryos did not provoke any malformations.

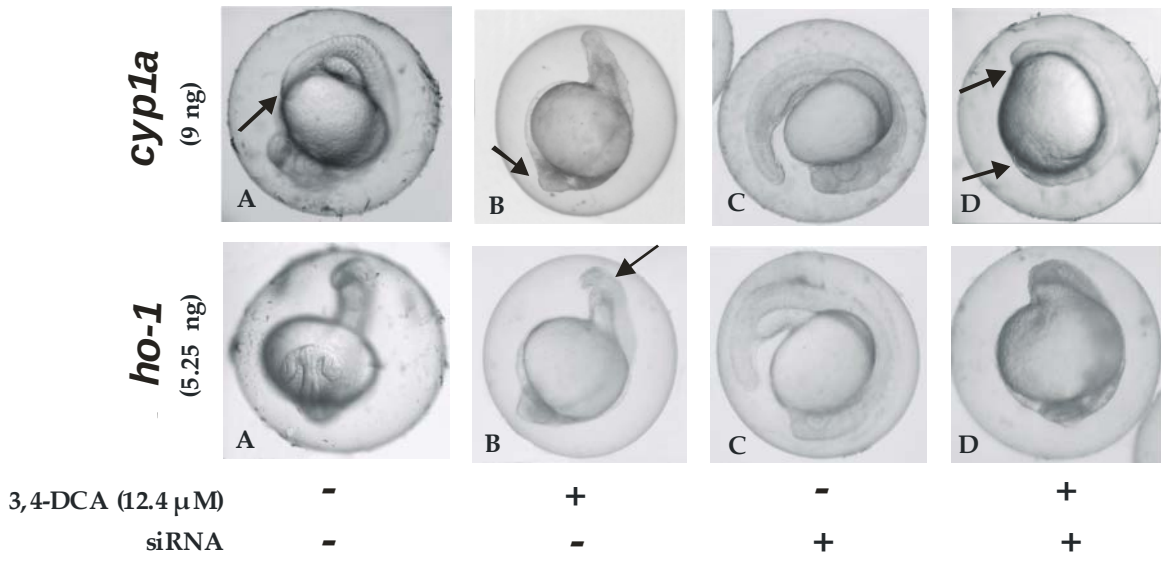

Figure 3.17 Injection of *cyp1a* siRNA and *ho-1* siRNA into zebrafish embryos increased severity of malformations caused by 3,4-DCA exposure (D). Injection of siRNA into non-exposed control embryos did not cause any phenotypical alterations (C). Arrows indicate prominent disorders of head and tail during embryonic development (A = non-exposed, non-injected control embryo, B = exposed, non-injected control embryo).

In order to silence *maft* mRNA, two different siRNAs were injected into embryos at different concentrations (si-*maft* no.62: 6.3 ng and 3.15 ng; si-*maft* no.66: 10.2 ng and 5.1 ng, table 2.4). For both siRNAs, no mRNA repression was observed (figure 3.16, si-*maft* no.66). In accordance with these findings, no alterations in frequency or severity of developmental aberrations in 3,4-DCA-exposed embryos injected with si-*maft* were found.

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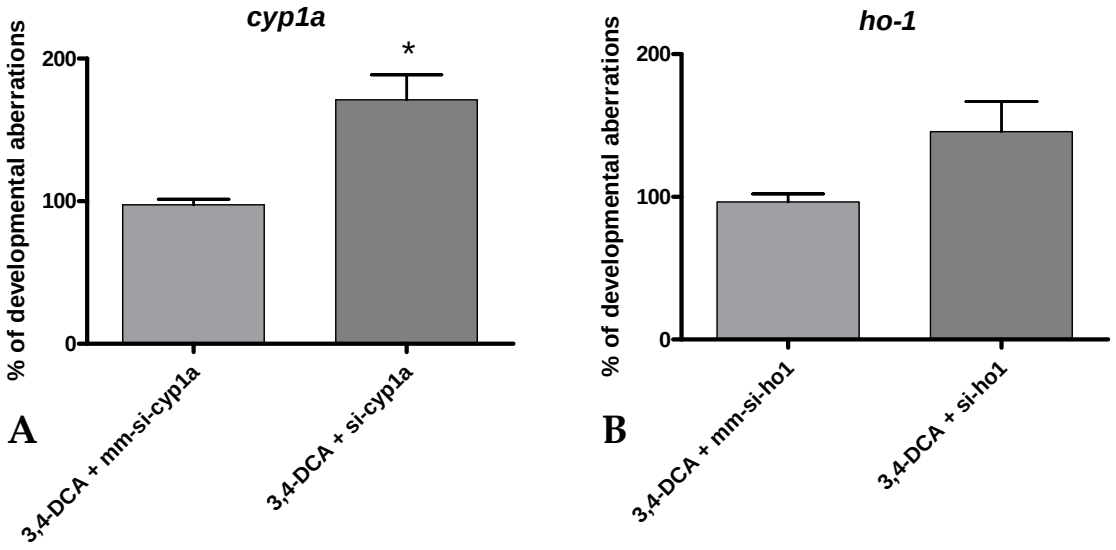

Figure 3.18 Repression of the genes *cyp1a* (A, n=3) and *ho-1* (B, n=4) in embryos exposed to 12.4 µM 3,4-DCA caused an increase in the frequency of developmental disorders (right bars). The frequency of developmental disorders was calculated by comparison to non-injected embryos exposed to 3,4-DCA (= 100 %). Mismatch-siRNA injections did not alter frequency or severity of 3,4-DCA-dependent malformations (left bars; mm-si-*cyp1a*, n=3; mm-si-*ho-1*, n=3) (*significantly different if compared to embryos injected with the corresponding mismatch-siRNAs in an unpaired, two-tailed t-test, $p < 0.05$).

CHAPTER IV

DISCUSSION

In this thesis, toxicant-sensitive expression of genes in zebrafish embryos exposed to a model compound was investigated on order to elucidate the potential of gene expression analysis to refine and extend conventional toxicity tests. RT-PCR and microarray techniques were used to screen for genes with toxicant-related alterations in their expression. The sensitivity of the analysis of differentially expressed genes was compared to the sensitivity of classical toxic endpoints of the embryo toxicity test (*Dar*T). Transient manipulations of mRNA levels of selected genes were performed to investigate the adaptive functions of these genes in response to the toxic impact of the model compound.

4.1 Identification of 3,4-DCA-sensitive genes

In the last decade, analysis of gene expression evolved as a promising approach to study the response of cells or whole organisms to a changing environment or stimulus. In toxicology, gene expression analysis offers the possibility to elucidate the molecular impact and the mode of action of a toxicant. Using microarray technology, 10 000s of genes can be analysed simultaneously, e. g. in order to establish gene expression profiles of toxicants. These profiles not only summarise and visualise the cellular reactions upon exposure of a compound; they may also provide a database of toxicant-sensitive genes for the prediction of unknown toxic effects of other compounds. Networks of gene expression that are common for different treatments might reveal substances of similar mode of actions or - vice versa - alternative modes of action. In consequence, gene expression analysis refines the significance of already existing toxicity test systems.

In ecotoxicology, i. e. in toxicology focussing on non-humans, the use of genomic technology is still in its infants. However, more and more studies are evaluating the molecular impact of compounds using microarray technology (Lettieri, 2006). Recent examples of the application of microarrays in ecotoxicology are dealing with the examination of gene expression profiles upon exposure to estrogens (Larkin *et al.*, 2003; Kishi *et al.*, 2006), polluted effluent water (Williams *et al.*, 2003), oxidative stressors (Girardot *et al.*, 2004), pharmaceuticals (van der Ven *et al.*, 2006) and other agents.

Different organisms, in particular fish (Larkin *et al.*, 2002; Ton *et al.*, 2003; Williams *et al.*, 2003; van der Ven *et al.*, 2006), but also insects (Girardot *et al.*, 2004), have been applied to study the impact of stressors on gene expression.

In this study, microarrays and RT-PCR were deployed to screen for chemical-sensitive gene expression in zebrafish embryos. On the one hand, these genes could indicate adaptive and toxic effects at sub-acute concentrations. On the other hand, the challenge was to gain insight into the modes of action by the analysis of altered gene expression. The thesis was focused on the identification of differentially regulated genes for the reference compound 3,4-dichloroaniline (3,4-DCA). 3,4-DCA was chosen due to its environmental occurrence as a pesticide metabolite and its usage as reference compound in the zebrafish embryo toxicity test (*Dar*T). Molecular responses to 3,4-DCA exposure have not yet been studied in fish and fish embryos. The identified 3,4-DCA-sensitive genes may contribute to the establishment of a battery of toxicant-sensitive genes in zebrafish embryos, which may indicate modes of action and chronic toxicity of chemicals in fish.

4.1.1 Microarray experiments

Although many effects of potentially hazardous agents might be manifested first on the protein level, they will finally – through cellular stress responses and interactions with signal transduction pathways - lead to changes at the gene expression level. While the analysis of proteins would better reflect the functional level, gene expression analysis is currently more advanced and better suitable for high throughput analysis.

A zebrafish oligonucleotide array, comprising more than 14000 zebrafish specific genes, was used for the analysis of the impact of 3,4-DCA exposure on gene expression during zebrafish embryonic development. Two different concentrations were investigated. Embryos were exposed to 3.1 µM 3,4-DCA, which corresponds to ¼ of the LOEC, and to 12.4 µM, which is the LOEC. By means of a significance analysis of microarrays (SAM), the zebrafish array did not reveal any significant alterations in gene expression in embryos exposed to 3.1 µM 3,4-DCA. This might be due to a low

induction or repression rate of genes upon exposure to sub-acute concentrations. Indeed, using samples of embryo exposed to 12.4 μM 3,4-DCA, SAM revealed a set of differentially expressed genes, but with a false discovery rate of 47%. The high false discovery rate indicates a high variability of hybridisation intensities on each slide, which hampers significance analysis. In particular, weak fluorescence signals may show a high variation and influence the analysis of significance. Therefore, 30 % quantiles with the lowest signal intensity in both channels of experimental and control hybridisations were removed from the analysis. Quantile filtering reduced the number of significant genes and the degree of variation. The false discovery rate decreased from 47 % to 12.8 %. The reduction of falsely positive detected genes upon quantile filtering is an indication for the high variability of genes of low hybridisation intensities and a rationale for the removal of data of low fluorescence intensity.

SAM revealed seven differentially expressed genes (5 induced and 2 repressed) in zebrafish embryos exposed to 12.4 μM 3,4-DCA. The identified genes exhibit different functions in biotransformation (*cyp1a*, *ahr2*), cell proliferation (*fzr1*) or basic cell function (*ferritin middle subunit*, *proteasome component c7-i*, *alpha-2-macrogobulin*), respectively. Most of these genes only showed a low fold-change of expression. A commonly applied cut off for the selection of differentially expressed genes identified by microarray is a fold-change of 2 (Xu *et al.*, 2006; van der Ven *et al.*, 2006). In the presented array experiments, only a few genes reached this cut-off. Thus, the overall low fold-change may explain the low number of identified genes and the conformation of exclusively those genes with either a high SAM score or a high fold-change of expression by RT-PCR (e.g. *fzr1* and *cyp1a*).

Three of the genes identified by microarrays were confirmed by quantitative RT-PCR to be concentration-dependent differentially expressed in an independent set of samples. The genes *cyp1a*, *ahr2* and *fzr1* were detected to be regulated even at concentrations below those that elicit toxic effects. The gene *cyp1a* was found to be significantly induced at 0.78 μM and above, *ahr2* was elevated at 6.2 μM 3,4-DCA. *fzr1* showed a significantly reduced expression at 3.1 μM and above. While RT-PCR indicated a significant induction of *cyp1a* at 0.78 μM 3,4-DCA and above, microarrays

did not indicate any differential expression at 3.1 µM. Furthermore, *fzr1* exhibited a significant repression at 3.1 µM revealed by RT-PCR, while microarray experiments did not show any significant differential expression of *fzr1* in embryos exposed to 3.1 µM.

Due to the exponential amplification, RT-PCR represents a very sensitive tool, which facilitates the identification of differences in transcript levels even at low abundance compared to microarray technology (Draghici *et al.*, 2006). The expression level of *cyp1a* at 3.1 µM might be too low to be detected by microarrays. Thus, the lower sensitivity justifies the usage of relatively high exposure concentrations and additional analysis of identified genes at lower concentrations by RT-PCR.

The low number of significant differentially expressed genes may also be biased from the selection of gene sequences on the 14 k microarray. The 14 k oligonucleotide is based on a rather early stage of the zebrafish genome sequencing and was established in October 2002. A large set of genes or ESTs that contributed to the preparation of this 14 k oligonucleotide set has been generated from a variety of untreated tissues or embryonic stages. Thus, they may not include genes induced by exposure to chemicals. Indeed, analysis of the genes available on the oligonucleotide array indicated that some important genes involved in stress response and anti-oxidative defence were not included. Furthermore, differential gene expression could be restricted to a specific organ or tissue of the embryo. Thus, alterations in expression might be masked by the overall expression of the gene in the whole embryo, which could hinder the identification of toxicant sensitive genes. The current test set-up does not allow for a spatial analysis of gene expression in embryos.

With respect to other reports on the utilisation of microarray technology, the confirmation rate of significant differentially expressed genes presented in this study is relatively low. Most of the microarray-confirmation studies presented in publications were conducted using RNA of the same pool which was already deployed for the microarray experiments (e.g. Kishi *et al.*, 2006). In this thesis, RNA of three independent samples was used for confirmation experiments in order to include

biological variation and obtain genes of robust toxicant-sensitive expression.

The low confirmation rate of differential expression elucidated by microarray and the differences in gene expression of replicated RT-PCR experiments are indicators of technical and/or biological variances. To overcome limitations based on technical variances, in particular if microarrays are not deployed for screening but as a diagnostic tool, the number of replicates needs to be increased. The use of a common reference RNA (Sterrenburg *et al.*, 2002) might also reduce variations in microarray analysis. In a common reference design, both cDNA of experimental and of control samples are each hybridised together with a unique cDNA derived from a large pool of RNA used for all analyses. Using this approach, variances in hybridisation efficiencies are balanced by the common reference RNA. Other advantages are that a dye swap can be omitted and that variations within controls are now visible.

The low number of differentially expressed genes is in part also attributable to low signal intensity for weakly expressed genes. Improvement of signal intensity could be achieved by using linear amplified labelled RNA for hybridisation instead of cDNA. In this approach antisense RNA is synthesised by *in vitro* transcription of double-stranded cDNA templates, harbouring a polymerase promoter sequence. Since the *in vitro* transcription to aRNA represents a linear amplification step prior to hybridisation, it preserves the ratios between the abundance of transcripts in different samples.

To overcome limitations based on biological variances, which disturb the identification of valid differential expression, in this study, RT-PCR using samples of at least three independent samples was performed. RT-PCR also indicated high variations of gene expression levels. Since the response of the investigated genes to treatment was characterised by a conserved trend, significant differences in expression were found upon normalisation and standardisation of the data sets.

4.1.2 Expression analysis of genes not included in the array

Selected genes with important functions in stress or toxic responses, but not included in the microarray, were screened additionally by RT-PCR. Twenty candidate genes, which had been shown to be either up- or down-regulated in processes related to the response to chemical exposure, were analysed. With the exception of *hsp70*, *nrf2*, *mafg1* and *2*, *maft*, *gstp1* and *2* (Lele *et al,.* 1997; Rabergh *et al.,* 2000; Martin *et al.,* 2001; Mattingly & Toscano, 2001; Yamazaki *et al.,* 2002; Suzuki *et al.,* 2005), the selected genes had not been studied with respect to chemical exposure or heat stress in zebrafish embryos or larvae before.

Conventional RT-PCR revealed an induction of the genes *nrf2, mafg1, maft, hsp70* and *ho-1* in embryos exposed to 3,4-DCA. None of the other genes showed alterations of transcript levels. Differential expression was also analysed by quantitative RT-PCR. Three of the previously identified genes (*nrf2, maft* and *ho-1*) showed a significant, induced expression in zebrafish embryos exposed to 3,4-DCA. These genes are involved in the regulation of phase 2 processes and in stress responses, particularly the mitogen-activated protein kinase (MAPK) or antioxidant defence pathways (Kobayashi *et al.,* 2002; Pratt *et al.,* 2002; Martin *et al.,* 2004; Motohashi & Yamamoto, 2004; Takagi *et al.,* 2004).

The 20 candidate genes have been selected from reports on changes in their transcript levels in various test system. Some of the selected genes (e.g. *hsp70, gstp1* and *2*) have already been reported to be differentially expressed in zebrafish by exposure to chemicals, but did not show significant alterations in expression in embryos exposed to 3,4-DCA. The genes *hsp70*, *gstp1* and *2* may not be affected by 3,4-DCA exposure. However, exposure duration and sensitivity of exposure, as well as the use of different stages, could be responsible for the failure to induce gene expression in the *Dar*T (*Danio rerio* embryo test). Maximum induction of *hsp70* was reported at 1 hour of heat shock at 37°C and decreased to control levels at later stages (Lele *et al.,* 1997). Blechinger *et al.* (2002) found significant *hsp70* induction in 72 hpf early larvae exposed to cadmium chloride for 96 hours. Induction of *gstp1* and *2* upon exposure to diethylmaleate (DEM)

was observed in 5 days old hatched larvae (Suzuki *et al.*, 2005). In contrast, in the *Dar*T set-up, embryos are continuously exposed from fertilisation for 48 hours, i.e. for the complete embryonic development until hatching. Furthermore, genes affected by xenobiotics may also show their differential expression only in certain tissues. To detect these differences, the localisation of the gene expression by in situ hybridisation could be used (Coverdale *et al.*, 2004). However, these data are difficult to quantify, making this technique rather unsuitable for screening.

4.1.3 Sensitivity of gene expression at different life stages and with respect to toxic endpoints

Differential gene expression in zebrafish embryos exposed to 3,4-DCA was found below toxic concentrations. Significant gene inductions or repressions in embryos were 2 - 16 fold below the LOEC for acute toxic effects (12.4 µM). The highest sensitivity was observed for *cyp1a* expression. However, if compared to sub-lethal chronic effects of 3,4-DCA ($LOEC_{ELST}$ = 0.22 µM), gene expression in the *Dar*T is 3.5-28.5 fold less sensitive.

In post-hatch sac fry stages differential expression already occurred at exposure concentrations of 3,4-DCA below those needed to induce alterations in embryos. Increase in transcription was observed in 5 dpf larvae for *ahr2*, c*yp1a, maft, nrf2* and *ho-1* at concentration of 0.22 to 0.93 µM DCA and above. Differential expression of these genes was also detected in 30 day old juveniles exposed to 0.46 µM 3,4-DCA and above. For *fzr1*, a slightly reduced gene expression was only detected in 30 dpf embryos exposed to 0.93 µM 3,4-DCA. Thus, in contrast to gene expression in embryos, post-hatch sac fry stages show differential gene expression in the same concentration range as toxicity is observed by classical toxic endpoints of the early life stage test.

The similar trend for gene expression changes in embryos, post-hatch sac fry and juvenile stages indicates that appropriate cellular and molecular signalling pathways for the response to chemicals or stress are already established in embryonic stages. The lower sensitivity of gene expression in pre-hatched embryos might be associated with a

lower sensitivity compared to larval stages, but may also be explained by the duration of exposure and/or increased bioaccumulation of the test compound.

Furthermore, sensitivity could be biased by the gene set that was used in the analysis. Since the genome of zebrafish is estimated to contain between 26600 and 48200 genes (www.ensembl.org/Danio_rerio/index.html), many possible target genes could be missed by the 14 k array and the RT-PCR-based analysis of selected candidate genes. Upon completion of the zebrafish genome project the number of potential target genes that can be analysed by microarray technique will have substantially increased and may lead to the identification of genes whose expression is affected by lower exposure concentrations.

One of the goals of the Gene-*Dar*T project, in which this study was embedded, was to deploy gene expression in zebrafish as a potential endpoint to indicate or predict chronic toxicity. In this respect, the differences in sensitivity at different stages or exposure periods are important. The use of later stages could be advantageous, due to enhanced sensitivity or better correlation with chronic effects. However, the current legislation – at least in Germany - limits the application of the embryo test as an alternative method to pre-hatched stages (BMBF, 2001). The German legislation is based on the EU Directive 86/609/EEC, which regulates the experimental use of non-human vertebrates. According to this directive, embryonic stages are accepted as alternatives to animal experiments (BMBF, 2001). However, in particular for fish there is a controversial discussion on the definition of the period of embryonic development. Some scientists define post-hatch stages as part of the embryonic development continued after hatching ("eleuthero embryo", Balon, 1999; Urho, 2002). In a statement for the amendment of the EU directive 86/609/EEC this view was supported (EFSA, 2005).

Due to this controversial discussion, there are different interpretations in the national legislation for the EU directive. Currently, no general definition exists for the embryonic period that can be accepted as in vitro alternative. The completion of the amendment of the EU directive may lead to a harmonisation of national practices.

However, this may not necessarily mean that the usage if post-hatched stages will be considered as *in vitro* method. At least the use of post-hatched stages could be seen as a refinement - the potential suffering of animals may be reduced in a test system using reduced exposure time in early life stages.

4.1.4 Mechanism of 3,4-DCA effects deduced from gene expression analysis

The 3,4-DCA-sensitive genes are indicative for adaptation by biotransformation (*cyp1a, ahr2*) and cellular stress response (*nrf2, maft, ho-1*) as well as the interference with the cell cycle control (*fzr1*). CYP1A is a cytochrome P450 isoenzyme that is part of the phase 1 biotransformation of xenobiotics. The strongest activators of *cyp1a* are halogenated and non-halogenated polyaromatic hydrocarbons with planar orientation of the aromatic rings (Waller & McKinney, 1995). CYP1A is catalysing hydroxylation reactions to support the detoxification and excretion of substances from organisms (Riviere & Cabanne, 1987; Nebert *et al.,* 1989; Goldstein & Faletto, 1993). Its transcription is controlled by the Ah-receptor (AHR), a nuclear receptor which can bind polyaromatic and other substances (Bock, 1994; Waller & McKinney, 1995; Denison & Heath-Pagliuso, 1998). An AHR-ligand enters the cell and binds with high affinity to the intracellular receptor. Ligand-binding results in a conformation change, exposing a nuclear translocation site. The complex is then translocated into the nucleus. Here, the receptor part dimerises with a nuclear protein called ARNT. This dimer exhibits a high DNA binding affinity. The complex of ARNT and AHR plus ligand binds to a specific promoter region (Bock 1993), the so-called dioxin responsive element (DRE). The DRE is located upstream of AHR responsive genes, e.g. phase 1 genes such as *cyp1a*. Binding of the receptor-ligand complex to this promoter region enhances the transcription of the corresponding downstream gene (Denison & Nagy, 2003). Like *cyp1a,* the expression of *ahr* is also regulated via DREs (Safe, 1995).

The induction of *cyp1a* expression upon exposure to 3,4-DCA supports the finding that also monoaromatic compounds, such as 2,3-diaminotoluene are able to induce *cyp1a* (Denison & Heath-Pagliuso, 1998). The 3,4-DCA-sensitive expression of *cyp1a* is in line with the metabolism pathways reported for (chlorinated) anilines. In zebrafish,

chloroanilines are metabolised to acetanilids (Zok *et al.,* 1991). It has been shown that CYP1A2 is responsible for the N-oxidation of arylamines with a subsequent *O*-esterification by *N*-acetyltransferases (King *et al.,* 1999). This pathway may also be active in zebrafish.

The stress response genes induced by 3,4-DCA in zebrafish embryos are involved in the induction of phase 2 detoxification enzymes and antioxidant proteins. The basic-leucine zipper protein p45 NF-E2 related factor2 (NRF2) is one of the major inducers of phase 2 detoxification enzymes (Motohashi & Yamamoto, 2004). Furthermore, heat shock proteins are induced via the NRF2 pathway (Kwak *et al.,* 2003), which may explain the induction of the heat shock protein and antioxidant enzyme *hsp32/ho-1* (Martin *et al.,* 2004). NRF2 is controlled via several transcriptional cofactors by MAPKs (mitogen activated protein kinases; Shen *et al.,* 2004). In the absence of an inducer, NRF2 is associated with the cytoplasmatic protein KEAP1 and is degraded by the ubiquitin-proteasome pathway (Itoh *et al.*, 1999). Translocation of NRF2 to the nuclei upon exposure to xenobiotics or oxidative stress leads to the dimerisation with so-called small MAF proteins (v-maf musculoaponeurotic fibrosarcoma oncogenes; Motohashi & Yamamoto, 2004), of which the induction of gene expression was also observed in this study. NRF2/MAF-protein dimers are mainly targeting the antioxidant (ARE) and electrophile response elements (EpRE) of genes encoding for different phase 2 detoxification enzymes or enzymes of antioxidant functions and enhance their expression (Alam *et al.,* 1999). Furthermore, the promoter region of a maf gene exhibits a response element where NRF2/MAF heterodimers are able to bind (Katsuoka *et al.,* 2005). This indicates an auto-regulatory feedback pathway, which transcriptionally regulates small *maf* genes.

Recently the AHR- and the NRF-mediated pathway have been considered as two linked signalling ways in the response to external stimuli and in the regulation of biotransformation processes. In 2005, Miao and colleagues reported a transcriptional regulation of the transcription factor *nrf2* of phase 2 detoxification pathways by the AHR receptor. They demonstrated a direct binding of AHR to a novel xenobiotic response element, found in the promoter region of mouse *nrf2*. Furthermore, they used

the siRNA silencing approach to knock down *ahr* expression in a mouse cell line, which resulted in a loss of *nrf2* induction upon exposure to 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD).

To investigate a possible linkage of the activation of AHR2 and the induction of *nrf2* in zebrafish embryos, β-naphthoflavone (BNF) was used. BNF is a known AHR agonist. Like hPAH and PAH, BNF exhibits a high affinity of binding the AHR and subsequently inducing CYP1A. In this study, BNF also significantly induced expression of *nrf2* and its target gene *ho-1*. This observation indicates a possible activation of NRF2-regulated detoxification pathways by an AHR2-ligand complex-mediated signalling also for zebrafish embryos.

In contrast to *cyp1a*, *nrf2* and *ho-1,* the fizzy related protein 1 (*fzr1*) does not appear to be directly linked to the above described defence response. Its expression is not reduced by exposure to β-naphthoflavone and thus does not indicate a regulation linked to the Ah-receptor pathway. FZR1 is one of the key regulators of a large multisubunit complex, the anaphase-promoting complex or cyclosome (APC/C). This complex is catalysing the ubiquitination of cyclin B. A controlled degradation of cell cycle regulators like cyclin B1 is necessary to maintain the sequence of the different cell cycle phases. The proteolysis of mitotic cyclins A and B is essential for the cell's exit of mitosis (Inbal *et al.,* 1999; Raff *et al.,* 2002). 3,4-DCA might influence cell cycle control by repressing the transcriptional activity of FZR1. Decrease of *fzr1* transcription mediated by 3,4-DCA could result in a block of mitosis leading to toxic effects. However, functional analysis such as overexpression in exposed embryos is necessary to unravel the potential implications of *fzr*1 for toxicity.

In summary, the differentially expressed genes in zebrafish embryos exposed to 3,4-DCA indicate a regulatory network, which is mainly based on the Ah-receptor and may also involve the activation of MAP-kinases as nodal point (Figure 4.1). The induction of Ah-receptor-/MAP-kinase-regulated genes indicates a general defence response, which is likely to be activated by other chemicals as well. Its function could be related to detoxification processes leading to a more effective metabolism of

xenobiotics. However, they may also indicate toxic effects, as it can be anticipated that adaptive responses precede toxic effects or occur at concentrations close to those that elicit toxic effects.

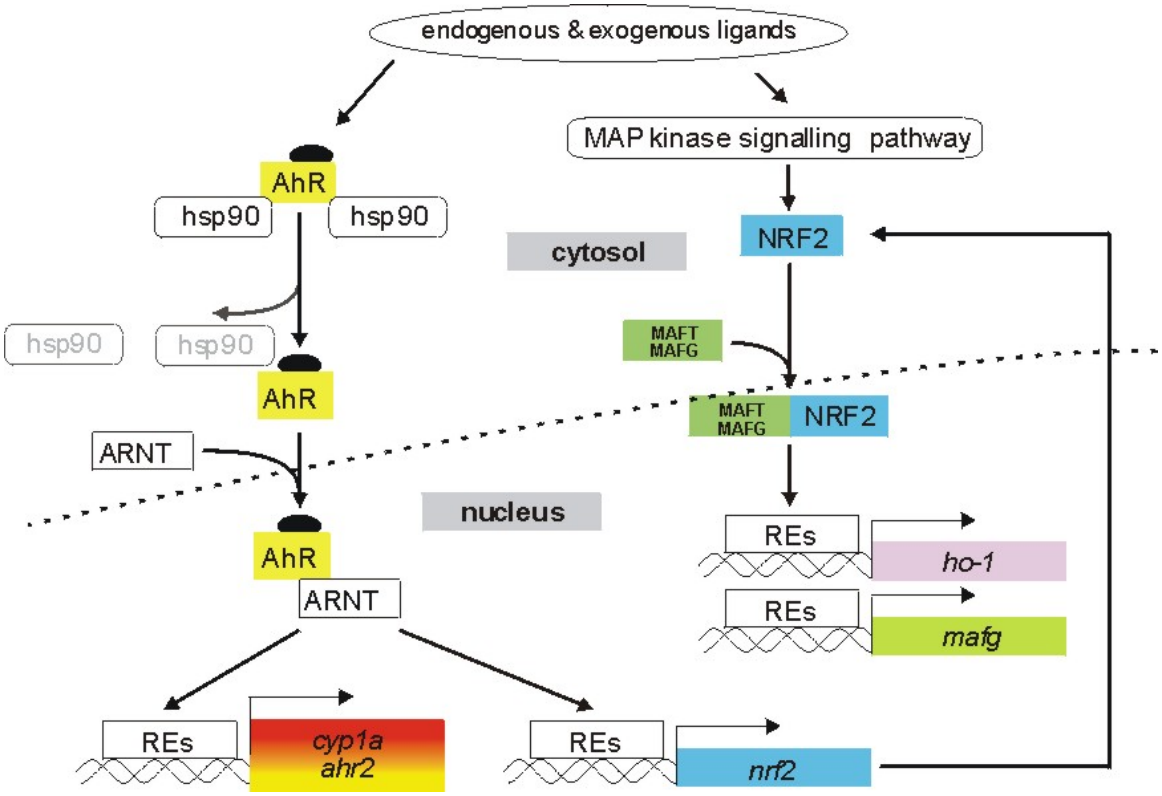

Figure 4.1 Illustration of signalling pathways that might be involved in the induction of genes in zebrafish embryos exposed to 3,4-DCA: *cyp1a* and *ahr2* transcription are enhanced via binding of 3,4-DCA to AHR2. Subsequently, the AHR2-ligand complex is supposed to act as inducer of several transcriptional activators (e.g. NRF2 and MAFT) of phase 2 biotransformation enzymes as well as antioxidant enzymes (e.g. HO-1). It is known that these enzymes are also mediated via MAP-kinase stress responses (modified from Rushmore & Kong, 2002; Denison & Nagy, 2003; Miao *et al.*, 2005; Katsuoka *et al.*, 2005; ARNT = aryl hydrocarbon receptor nuclear translocator, hsp90 = heat shock protein 90, RE = regulatory element).

4.2 Relevance of gene expression for toxicity

After the identification of whole genomes, one of the actual challenges in biology is the investigation of gene function. In developmental biology, gain- and loss of function studies are widely explored by knock out mutants, transient knock down or overexpression of genes. In toxicology, a limited number of studies have been performed using functional manipulations to investigate the relevance of gene

expression in the evolvement of toxicity (Teraoka *et al.,* 2003; Wang *et al.,* 2004; Cao *et al.,* 2005; Knorr-Wittmann *et al.,* 2005; Menendez & Lupu, 2005; Billiard *et al.,* 2006; Saito *et al.,* 2006). In the zebrafish model, in particular transient approaches to manipulate *ahr2* and *cyp1a* have been already reported (Teraoka *et al.,* 2003; Billiard *et al.,* 2006).

Chemical-sensitive gene expression may reflect the mediation of toxicity by affecting important cell functions. On the other hand, it may represent an adaptation of the organism to the toxicant, resulting in a higher detoxifying activity and increased resistance. It is also known that - depending on the toxicant - biotransformation activity might lead to higher toxicity of the metabolites. Thus, the consequences of altered gene expression for toxicity depend on the nature of the parent compound, its metabolites and the differentially expressed target genes. Although the functions of the genes identified in this thesis are known and indicative of adaptive responses, these roles had not yet been experimentally proven. Therefore, in this thesis, the role of differential gene expression for toxicity was evaluated by transient knock down and overexpression approaches.

mRNA manipulation of 3,4-DCA sensitive genes

The siRNA-mediated knock down of *cyp1a* in zebrafish embryos increased the toxicity of 3,4-DCA, while *cyp1a* mRNA overexpression decreased 3,4-DCA toxicity. The influence of altered *cyp1a* mRNA abundance on the impact of 3,4-DCA on zebrafish embryonic development suggests translation of the *cyp1a* gene during embryonic development. It has been discussed that *cyp1a* might be transcriptionally silenced in zebrafish embryos (Mattingly & Toscano, 2001). However, the findings of this study support other observations using morpholino-mediated gene knock down of c*yp1a* transcription (Teraoka *et al.*, 2003; Billiard *et al.*, 2006).

CYP1A induction leads to an enhanced detoxification of xenobiotics, however, metabolites of some hPAH and PAHs are of higher toxicity (Guengerich & Liebler, 1985; Payne *et al.*, 1987). Thus, whether CYP1A induction represents an adaptation depends on the target compound. Teraoka *et al.* (2003) found a repression of TCDD

toxicity in embryo injected with *ahr2* and *cyp1a* morpholinos, which indicates a mediation of TCDD toxicity by phase 1 biotransformation. In contrast to that, Billiard *et al.* (2006) demonstrated enhanced toxicity of BNF upon morpholino-mediated knock down of *cyp1a* in zebrafish embryos.

The observations of this study upon both siRNA-mediated knock down and mRNA overexpression of *cyp1a* in embryos exposed to 3,4-DCA support the adaptive and protective role of CYP1A. Induction of biotransformation enzymes such as CYP1A by 3,4-DCA would elevate the metabolic rate, enhance excretion and thus, reduce toxicity. Injection of mRNA would amplify such an effect. Thus, the effects of *cyp1a* mRNA manipulations indicate a potential metabolism of 3,4-DCA by CYP1A. However, a chemical analysis of potential metabolites would be necessary to support or reject this conclusion.

Overexpression of *ho-1* via mRNA injections significantly reduced toxicity of 3,4-DCA in exposed zebrafish embryos. In contrast, siRNA-mediated knock down of *ho-1* revealed a higher toxicity of the substance. However, statistically significant difference could not be demonstrated for overexpression effects but may be achieved by increasing the replicate number. The effects of *ho-1* siRNA and mRNA injection may be explained by the protective function of the corresponding protein. The enzyme heme oxygenase 1 (HO-1) is involved in the response of the cell to various external stimuli (Choi & Alam, 1996), including agents causing oxidative stress (Maines, 1997). HO-1 is known to protect cells from oxidative stress via the heme degradation products bilirubin and biliverdin (Mazza *et al.*, 2003). The 3,4-DCA could induce *ho-1* via reactive oxygen species (ROS) caused cell damage and oxidation of membrane lipids. However, an induction of ROS by 3,4-DCA has not yet been demonstrated.

Ho-1 is also induced by heat shock and therefore also claimed as heat shock protein (HSP32; Chen *et al.*, 2000). Whether HO-1 is able to function as chaperonin and thus, remove or compensate misfolded proteins remains unclear. On the other hand, the degradation of heme, catalysed by HO-1, leads to the generation of carbon monoxide and iron, which are biologically active (Maines, 1997; Jin & Choi, 2005). The secondary

products of this reaction are likely to possess adaptive functions and might affect further downstream genes involved in adaptation.

Overexpression of the phase 2 transcription factor *nrf2* (nuclear factor E2 p45-related factor 2) had a protective effect in zebrafish embryos exposed to 3,4-DCA, since mRNA injections decreased toxicity. First investigations, using two replicates of siRNA injection, revealed enhanced toxicity in embryos with repressed *nrf2* transcript abundance. Additional experiments on siRNA-mediated *nrf2* silencing are needed to confirm the results and to pursue an analysis of statistically significant alterations. NRF2 is a transcription factor and supposed to mediate adaptation to toxicity through other genes downstream of its signalling cascade such as phase 2- or stress response genes. Overexpression of *nrf2* is supposed to increase the level of downstream detoxification enzymes and thus protect 3,4-DCA-related toxicity. So far, the observations made upon *nrf2* manipulation are in line with the protective function of *ho-1* expression, since induction of *ho-1* is known to be regulated by NRF2 action. Whether alterations in transcript abundance of down stream genes (e.g. *ho-1*) are detectable upon *nrf2* manipulation remains to be investigated.

The protein FZR1 is involved in the regulation of mitosis. *fzr1* transcription is repressed by 3,4-DCA. Overexpression of *fzr1* was performed in order to rescue *fzr1* repression by 3,4-DCA and to investigate the relevance of *fzr1* repression for 3,4-DCA toxicity. Embryos injected with *fzr1* mRNA did not show any changes in the frequency of malformations. However, by RT-PCR, enhanced *fzr1* transcript levels could not be detected. This failure is unlikely to be caused by incorrect synthesis of the mRNA. However, the DNA clone used for mRNA synthesis has not yet been sequenced in order to detect potential mutations. Higher injection concentrations might enhance *fzr1* mRNA abundance, but may also cause unspecific toxic effects in the embryos.

RT-PCR did not indicate any gene silencing effects in zebrafish embryos by injection of of two siRNAs against distinct mRNA sites of *maft*. Not every designed siRNA may silence the mRNA of a gene of interest (Liu *et al.*, 2005; Dodd *et al.*, 2004; Ambion *siRNA target finder:* http://www.ambion.com/techlib/misc/siRNA_finder.html).

Therefore the design of at least three siRNA sense and antisense templates is recommended. Liu *et al.* compared the efficiencies of three siRNAs, which targeted three distinct sequences of the *no tail* mRNA. RT-PCR experiments and changes of the *ntl* phenotype revealed strong variations of effective knock down of different siRNA target sites. Therefore, they concluded that the selection of an appropriate target site might be one of the most important steps for successful siRNA-mediated knock down of genes.

In this study, three target sequences for each gene were selected. In order to evaluate the relevance of *maft* expression for the evolvement of 3,4-DCA-toxicity in zebrafish embryos, silencing of *maft* might be achieved upon injection of siRNA against an alternative target site.

Control injections

In order to ensure specificity of manipulated mRNA abundance appropriate negative controls need to be performed. A possibility to confirm specific gene manipulation, which is particularly applied in developmental biology, is the rescue approach. Knock down and overexpression studies are conducted in parallel and the observed impacts of both actions are abolished or rescued by co-introduction. The limiting step of this method is the careful adjustment of concentrations for both antisense and sense RNA. An alternative approach is the injection of mismatch-siRNAs (mm-siRNAs) against a mixed sequence of the target mRNAs. Unspecific siRNAs are not supposed to specifically inactivate transcription of target genes. Neither injection of mm-siRNA for *cyp1a* nor *ho-1* into zebrafish embryos caused a suppression of mRNA abundance, confirming the specifity of the designed siRNAs.

Appropriate negative controls have also been established for the overexpression studies of *cyp1a, ho-1* and *nrf2*. The utilisation of a mismatch-mRNAs could be one possibility, but would require different controls for each gene. Since the production of mm-mRNA is more laborious than of mm-siRNA, a different approach was chosen. Total mRNA, purified from total RNA of zebrafish embryos, was used as control for mRNA injection. Total mRNA consists of only very low concentrations of the target

mRNA and is thus unlikely to cause specific effects. Therefore, unspecific effects of mRNA could be indicated by injection of total mRNA. It was observed that introduction of total mRNA neither changed mRNA abundance of *cyp1a, ho-1* or *nrf2*, nor influenced the frequency of developmental disorders of exposed embryos.

This is the first time that both siRNA-mediated knock down and mRNA overexpression of genes have been used for studying gene function in response to toxic impacts in zebrafish embryos. Silencing of two genes involved in cellular response to exogenous impact (*cyp1a* and *ho-1*) led to enhanced toxicity of the polar narcotic 3.4-DCA, while enhanced transcript abundance by mRNA injection of three genes (*cyp1a, ho-1* and *nrf2*) caused a significant decrease of toxic effects in the embryo. The alterations of 3,4-DCA-toxicity upon suppression and overexpression of mRNA transcripts support the adaptive and protective role of the genes *cyp1a, ho-1* and *nrf2*, since these genes were also found to be upregulated in embryo toxicity tests. Antagonistic effects by knock down and overexpression of *cyp1a* and *ho-1* verify the specificity of functional manipulation and the effects on the toxicity. The results of this study demonstrate that functional gene analysis can be utilised to identify gene function and mechanisms involved in the response to exposure of chemicals in zebrafish embryos.

CHAPTER V

CONCLUDING REMARKS & FUTURE DIRECTIONS

This study represents one of the first examples for microarray analysis of gene expression changes in whole zebrafish embryos exposed to a toxic chemical. Previously published investigations using microarrays in zebrafish embryos were focussed e.g. on the analysis of hypoxia (Ton *et al.*, 2003), the characterisation of differences between embryonic stages (Lo *et al.*, 2003) or the analysis of the interference of cyclopamine with the sonic hedgehog genes (Xu *et al.*, 2006).

It was shown that analysis of gene expression is able to indicate mechanistic information, which would not have been revealed by the classic toxicological endpoints of the zebrafish embryo test (*Dar*T). The detected set of genes indicated an involvement of Ah-receptor-regulated pathways as a likely adaptive response.

With respect to the reference compound 3,4-DCA, analysis of gene expression in embryos exhibited a higher sensitivity (up to 16 fold) than analysis of survival and developmental disorders in the embryo test. The enhanced sensitivity of gene expression was also observed for other chemicals. In co-operations with ECT Oekotoxikologie GmbH Flörsheim (M. Weil, Dr. K. Duis), genes identified and analysed in this thesis were investigated for differential expression in zebrafish embryos exposed to triazines, flavonoids, benzol- and aniline derivates and others. For many compounds, differential expression in embryos was found to be in the same range of sensitivity as toxic effects in an early life stage test (ELST; figure 5.1).

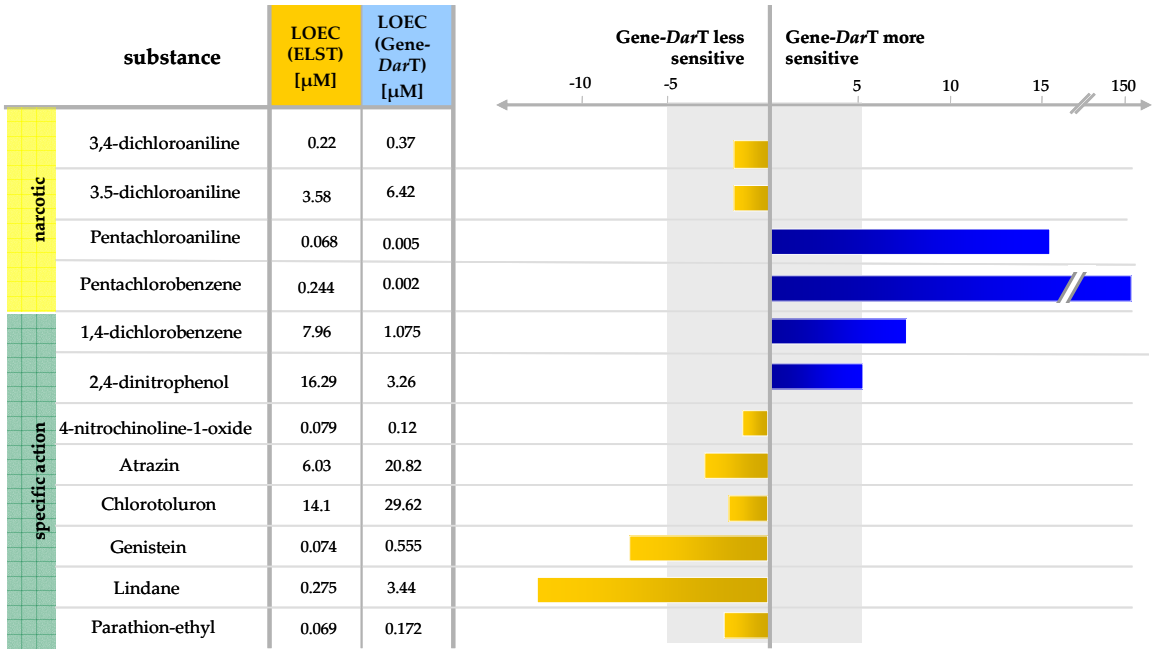

Figure 5.1 Relative sensitivity of Gene-*Dar*T, documented as LOEC Gene-*Dar*t/LOEC ELST. For 7 out of 12 substances, sensitivity of Gene-*Dar*T differs by a factor of ≤ 5 from sensitivity of the fish early life stage test (ELST). For 3 substances, Gen-*Dar*T is more than 5 fold sensitive than the ELST (Weil *et al.*, 2006).

The enhanced sensitivity of the embryo test offers the possibility that gene expression analysis in embryos might be used to indicate or even predict chronic effects. However, in order to be deployed as predictive model, the "Gene-*Dar*T" needs further development. Advances in the sequencing of the zebrafish genome will lead to the production of larger microarrays that may deliver additional differentially regulated genes with higher sensitivity. Larger microarrays may facilitate a clustering of differential gene expression into groups to identify specifically affected signalling pathways. Furthermore, it will be necessary to extend the analysis of gene expression to additional compounds of different chemical structures and mode of actions. In this way a set of genes could be established that is suitable for the analysis of many different chemicals.

The interpretation of (toxico)genomic data is often difficult without knowledge of the precise function of differentially expressed genes. In toxicology, particularly the knowledge of the gene function with respect to adaptation or mediation of toxic effects is of interest.

Gain- and loss of function studies, as applied in this thesis, represent promising tools to gain insight into molecular mechanisms upon exposure to toxicants. Both types of studies are already commonly used in molecular biology, especially in developmental biology. In parallel to methodic advances, their utilisation in toxicology just arose a few years ago. Morpholino antisense oligonucleotides- and – with limitation concerning the application in certain model organisms - RNA interference (RNAi)-mediated suppression of genes were applied to assess the relevance of gene expression for toxicity. This thesis represents one of the few studies reporting the utilisation of RNAi in zebrafish embryos. To my knowledge, it is the first application of RNAi in the context of (eco)toxicology in zebrafish.

Overexpression and siRNA-mediated knock down studies were performed to investigate the relevance of differentially expressed genes for the toxicity of the model substance 3,4-DCA. The significance of the adaptive responses of *cyp1a* and *ho-1* observed in this study may be limited to the applied toxicant. It is obvious that a compound which is metabolically activated by CYP1A would lead to opposing effects.

In summary, these studies show the feasibility of the gain- and loss of function approach to evaluate gene function in the context of (eco)toxicogenomics.

CHAPTER VI

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CHAPTER VII

ANNEX

Table A.1 Primer sequences for RT-PCR (*primers of this gene were also used for quantitative RT-PCR)

candidate gene name	ncbi acc. number	bp	qRT-PCR amplific. efficiency	primer sequences (5´- 3´, forward and reverse primers)
ferritin, middle subunit	BQ783379	246	-	AGAGAGGTGGACGCATTGTC TGGAGAGGTTAGTGATGTGGTC
proteasome component c7-1	BI710610	220	-	CAAACAGACGGCAAAACTGAC GATGGAGAGCGTGAGGAAAG
ferritin, middle subunit	BG892155	204	-	GCAGTGTGCTCTTCATCTGG TACTCCGCCATCCTGTTGTT
*aryl hydrocarbon receptor 2 (ahr2)**	NM_131264	232	95%	TACAGACTTCCAAAGAGGCACAC TGACATCCCAGATACCAGATTG
*cytochrome P450 1A (cyp1a)**	AB078927	211	84%	ATTCATCCTTCCTTCCCTTCAC ACCTTCTCGCCTTCCAACTTAT
*fizzy-related protein**	AW173921	203	92%	TTTTGGAACACTCTCACAGCAC ATTGCCAGATAAAGCACCCTAT
alpha-2-macroglobulin	BI326783	260	-	TCTGTTCTTGGAGTGGTGAATG TGAAACGAAGGATTTTGCTGAT
*transcription factor NF-E2 p45 related factor, nrf2**	AB081314	272	88%	GTTTGTCCCTAGATGCAAGTCC TCTTCAGCTTGTCTTTGGTGAA
*transcription factor, mafG1**	AB167540	215	76%	GGGGATCCAGGTGGAGAAGCTCGCCT GGCTCGAGCATTATGACCGTGCTTCTG
transcription factor, mafG2	AB167541	519	-	CATGACGACCACTAATAAAGG CTACTAAGACCTGGCGTCG
*transcription factor, mafT**	AB167543	210	82%	GAGGGACCAACAACATTTCAG ATTCAGACCAATCACAGCACAA
Pi-class glutathione S-transferase 1 (gstp1)	BC083467	711	-	CTAGGAGCAGCTTTGAAACGCAC CGTTGTTGGAGAATGTTGTACCGACG
Pi-class glutathione S-transferase 2 (gstp2)	AB194128	364	-	CACTCTCACATACTTCGCTATC AATATTTTCAAATGGTTTGAACTC
UDP-glucuronosyl-transferase (ugt)	CD759765	343	-	ACCTACTGGGATTTTGAGTTTCC GTGAGTGATGATGCTCTGGTTT
major vault protein (mvp)	BC049344	537	-	AGAAGAAGAGGAACGGGAAAGT AGCAGACAGATGGTTTTGATGAC
multi drug resistance protein (mrp)	AL591370	502	-	CTGAGAAGGGTGTGATAAAGGTG AGAAGAGAGGTGAAATCCAGTCC
*heme oxygenase (decycling), ho-1 (hsp32)**	NM_199678	214	-	GCTTCTGCTGTGCTCTCTATACG CTCTCAGTCTCTGTGCATATCG
*heat shock protein (hsp 70)**	AF006007	205	101%	GCATGGTGAACCACTTTGTGGAA CCCTGAAGAGGTCGAGCAAG
binding protein (bip)	BC063946	271	-	TCGTGGTGTCCCACAGATCGAGG ATCCCCGATCTGGTTCTTCAGG
topoisomerase 2	BQ078151	100	-	AGTTCATCACCCCTATCATCAAG GTGCTACTCTGCTTCTCTTTCCA
myosin light chain 2 (mlc2)	AF081462	376	-	CGGTATCATCAGCAAAGACG CTCCTTCTCCTCTCCGTGTG

Table A.1 (continued)

candidate gene name	ncbi acc. number	bp	qRT-PCR amplific. efficiency	primer sequences (5′- 3′, forward and reverse primers)
creatine kinase	BC056706	279	--	TGTCAAGCTGATGGTGGAGATGG AAGATGTTACCGACTTAATAGG
cytochrom-c-oxidase subunit I (cox)	NC_002333	460	-	TCTGACTTCTTCCACCCTCA GGAGATAATGCCGAATCCTG
enolase	NM_200695	290	-	AGCACAGAAATTCAGGTGGTGG TTGGCCAGACGCTCTGAACGGC
glutamate-cysteine ligase (gcl)	BC044532	296	-	TGGCAGGAGCTGGAGAGTCTGG ATGGAGTAGCGCAAAACCC
phosphoglycerate kinase 1 (pk1)	BC046026	196	-	TCTAGACTGTGGTCCTGAGAGC AGTGTCCCACTTGGCACAGCAGG
Ca2+ ATPase	BC051779	320	-	TTGGTGCCTATGTGGGTGCTGC AGTCAACCTGAAAATCATAGGC
beta actin (cytoplasmatic)	AF057040	471	-	GCCAACAGAGAGAAGATGACACAG CAGGAAGGAAGGCTGGAAGAG
*beta actin (cytoplasmatic)**	AF057040	216	99%	GAAATCACCTCTCTTGCTCCTTC GTTGGTCGTTCGTTTGAATCTC
cyclophilin A	NM_212758	242	-	GACTTCACAAACCACAATGGAA CCAAAGCCCTCTACTTTCTTGA

Table A.2 Primers used for the identification of successfully transformed E. coli ("colony PCR").

gene name	ncbi acc. number	vector	bp	primer sequences (5′- 3′, forward and reverse primers)
Fizzy related protein 1 (fzr1)	BC048038	pCMV-SPORT 6.1	249	ATTACAAAATAAGCGGCGAAGC GTGCGACAAAACTGGAGGTATC
NF-E2 related factor2 (nrf2)	BC045852	pCMV-SPORT 6.1	496	TTCGGGGTAAGACACACTAAAGA GTGCGACAAAACTGGAGGTATC
Cytochrome P450 1a (cyp1a)	NM_131879	pCMV-SPORT 6.1	409	GAAAGATTGGTCACGGTTATCG TAAAGGTTCAGAGGTGGGGTAA
Universal M13 (-20)	-	-	depending on vector and inserts	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC

Table A.3 Primers used for confirmation of siRNA-mediated gene knock down by RT-PCR. Primer location is upstream of the siRNA binding site.

gene name	ncbi acc. number	si target seq. no.	bp	primer sequences (5′- 3′, forward and reverse primers)
No tail (ntl)	AB088068	13	254	AAGACTGGGAGACGAATGTTTC TTGGAGAGTTTGACTTTGCTGA
Cytochrome P450 1A (cyp1a)	AY398333	10	296	GGTCCGATTTCTGTGTCTGAG AACTCTTCGCCCTGTTTTAGG
NF-E2 related factor2 (nrf2)	AB081314	54	272	GTTTGTCCCTAGATGCAAGTCC TCTTCAGCTTGTCTTTGGTGAA
V-maf musculoaponeurotic fibrosarcoma	AB167543	66	210	GAGGGACCAACAACATTTCAG ATTCAGACCAATCACAGCACAA
Heme oxygenase 1 (ho-; hsp32)	NM_199678	30	685	GCTGAAAACACACAACTGATGC CCACTCCTAATGCGAACTGAA

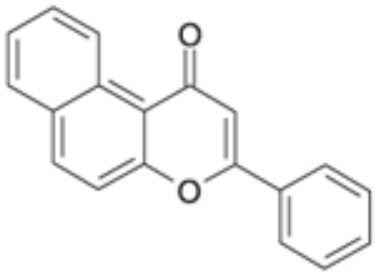


Figure A.1 Chemical structure of the AHR-agonist β-naphthoflavone (BNF).

Buffers, Reagents and Media

A.1 Embryo test medium

stock (1L)

0.75 mM $NaHCO_3$ (Merck)	2.59 g/L
0.08 mM KCl (Merck)	0.23 g/L
0.5 mM $MgSO_4x7H_20$ (Merck)	4.93 g/L
2 mM $CaCl_2x2H_20$ (Merck)	11.76 g/L

A.2 Stock solution of 3,4 dichloraniline (Fluka, 50 mg/L)

- solve of 12.5 mg 3,4-DCA in 250 ml of embryo test medium by stirring and heating at 30-40°C for 1 h
- 20` of ultra sonic
- 2-3h stirring and heating at 30-40°C
- additional 20` of ultra sonic
- store at dark at 4°C

A.3 Reverse transcription of RNA to first strand cDNA

- reaction mixture

10 µl DNA digested RNA
2 µl Oligo dT primer (10 µM, Invitrogen)

4 µl 5X RevertAid RT reaction buffer (MBI Fermentas)
0.5 µl ribonuclease inhibitor (recombinant, 40 U/µl, MBI Fermentas)
2 µl 10 mM dNTP mix (5 mM each, MBI Fermentas)
1.5 µl deionised water, nuclease free

1 µl RevertAid reverse transcriptase (200U/µl, MBI Fermentas)

20 µl per RNA sample

A.4 Polymerase chain reaction (RT-PCR)

- 10X RT-PCR buffer

  500 mM Tris/HCl (pH 9) (Serva)
  15 mM Magnesium chloride (Sigma)
  150 mM $(NH_4)_2SO_4$ (Sigma)
  1% (v/v) Triton X-100 (Merck)
  (store in aliquots at -20°C)

- reaction mixture

  5 µl 10X RT-PCR buffer
  1 µl 10 mM dNTP mix (5 mM each, MBI Fermentas)
  0.2 µl taq polymerase (5U/µl, Promega)
  40.8 µl deionised water, nuclease free
  1 µl primer forward (20 µM, Invitrogen)
  1 µl primer reverse (20 µM, Invitrogen)

  ---

  1 µl cDNA sample

  50 µl per cDNA sample

- TAE buffer (50X, 100ml)

2M Tris base (Roth)	24.2 g
0.5 M sodium EDTA (Serva electrophoresis)	1.86 g
acetic acid (Merck)	5.71 ml

  - add just to pH 8.0
  - store at room temperature and dilute 50X for electrophoresis and preparation of agarose gels

- agarose gel

  1 – 2 % (v/v) agarose (Roth) in 1X TAE buffer
  ~ 2 µl of ethidium bromide (Serva, 1%)
  (alternative: staining of the gel in a bath with 0.001% (v/v) ethidium bromide)

- DNA loading buffer (5X)

  50% (v/v) glycerol (Sigma)
  1 mM EDTA (MP Biomedicals)
  0.1% (w/v) orange G (Sigma)

A.5 Quantitative polymerase chain reaction (qRT-PCR)

- reaction mixture

2.5 µl 10X qRT-PCR buffer (Taq PCR Core Kit, Qiagen)
3.5 µl magnesium chloride (Taq PCR Core Kit, Qiagen)
0.5 µl 10 mM dNTP mix (5 mM each, Taq PCR Core Kit, Qiagen)
0.5 µl FITC (1 µM, Bio-Rad)
0.05 µl SYBR green (1:1000 dilutions)
0.1 µl Taq polymerase (5U/µl, Taq PCR Core Kit, Qiagen)
15.85 µl deionised water, nuclease free
0.5 µl primer forward (5 µM, Invitrogen)
0.5 µl primer reverse (5 µM, Invitrogen)

1 µl cDNA sample

---

25 µl per cDNA sample

A.6 Microarray solutions

A.6.1 phosphate buffers

- phosphate buffer (KPO_4,1 M, pH 8.5 – 8.7)

- phosphate wash buffer

5 mM KPO_4, pH 8.0
80% (v/v) EtOH (VWR)

- phosphate elution buffer

4 mM KPO_4, pH 8.5 (diluted in MilliQ)

A.6.2 dye coupling reaction

- sodium carbonate buffer

0.1 M Na_2CO_3 (Merck), pH 9.0

A.6.3 removal of uncoupled dye

- sodium acetate

0.1 M NaOAc (Sigma), pH 5.2

A.6 (continued)

A.6.4 hybridisation

- 20X SSC solution (1L)

  175.3 g sodium chloride (Merck)
  88.2 g tri-sodium citrate dihydrate (Merck)

  - adjust pH to ~7with HCl

- hybridisation buffer

  50% (v/v) formamide (Merck)
  5X (v/v) SSC
  0.1% (v/v) SDS (Roth)
  0.1 mg/ml salmon sperm DNA (AppliChem)

A.6.5 wash solutions

- Wash solution I: 0.1% (v/v) SDS (Roth); 2X (v/v) SSC
- Wash solution II: 0.2X (v/v) SSC
- Wash solution III: 0.1X (v/v) SSC
- Wash solution IV: 0.05X (v/v) SSC

A.7 Cloning

A.7.1 Luria Bertani (LB) medium and plates

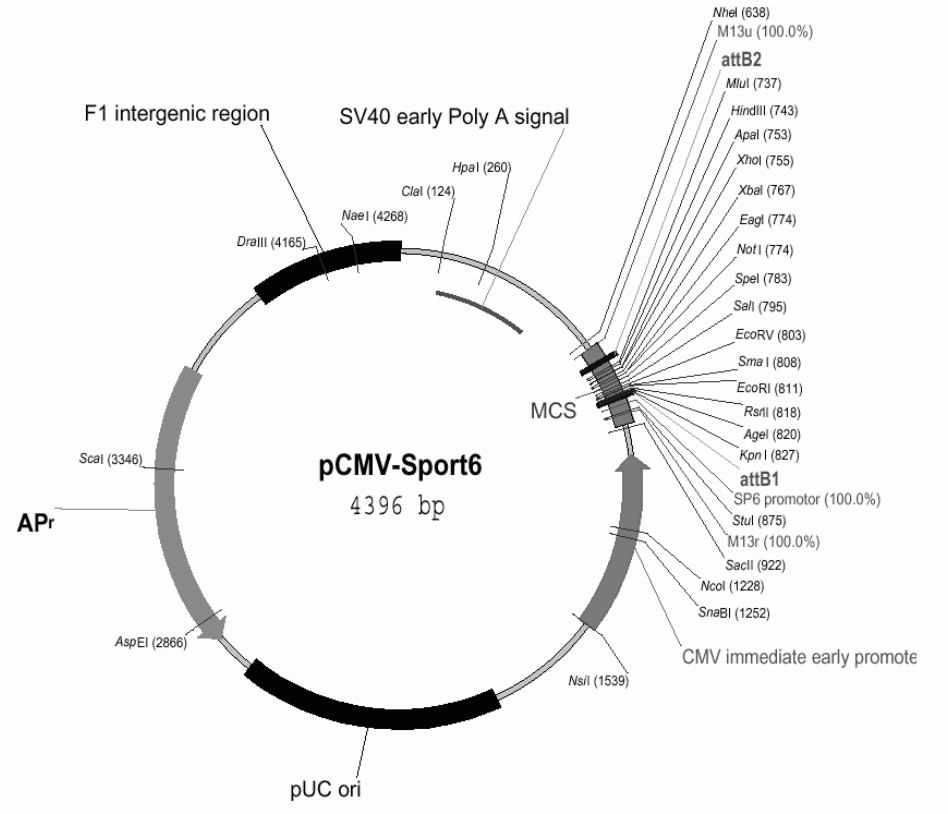
- Liquid medium (1L)

  1% (w/v) tryptone (Becton, Dickinson and Company)
  0.5% (w/v) yeast extract (Merck)
  1% (w/v) sodium chloride (Merck)

  - pH 7.0, autoclave, cool to 55°C, add ampicillin (50 μg/ml), pour into plates

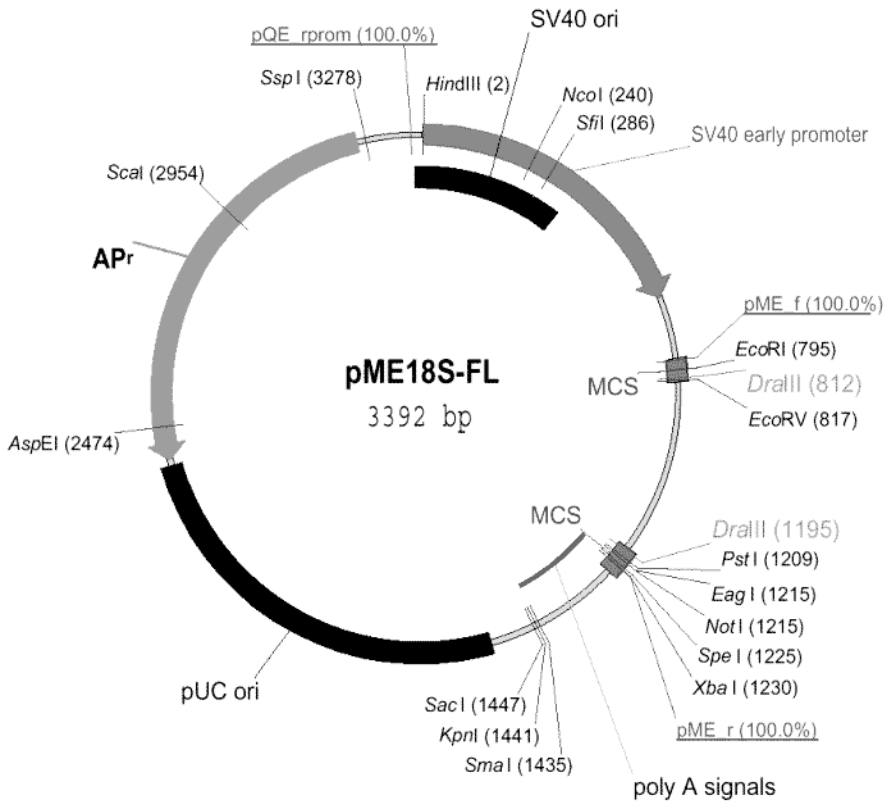
  - for LB plates, add 1.5% (w/v) bacto agar (Becton, Dickinson and Company)

A.7.2 Map of expression vector pCMV Sport 6.1 (Invitrogen)



modified from:
http://www.invitrogen.co.jp/products/pdf/custom%20_pCMVSPORT6.1library_man.pdf
more information at: http://www.rzpd.de/info/vectors/pCMV-Sport6.shtml

A.7.3 Map of expression vector pME 18S FL



modified from: http://www.rzpd.de/info/vectors/pME18S-FL.shtml
more information at: http://www.rzpd.de/info/vectors/pME18S-FL.shtml

A.8 Denaturing RNA agarose gel electrophoresis

- 10X MEN

  0.2 M MOPS (Sigma)
  0.05 M NaOAc (Sigma)
  0.01 M EDTA (MP Biomedicals)
  pH 7.0

- loading buffer

  50% (v/v) glycerol (Sigma)
  1 mM EDTA (MP Biomedicals)
  0.25% (w/v) orange G (Sigma)

A.8 (continued)

- gel

  1% (w/v) agarose (Roth) in bidestilled water, cook in microwave, cool to 60°C
  1X (v/v) MEN
  7% (v/v) formaldehyde (Merck)

- preparation of samples

  4 µl RNA/DNA ladder (MBI Fermentas)
  4 µl formamide (Merck)
  1.6 µl formaldehyde (Merck)
  0.8 µl 10X MEN
  0.4 µl ethidium bromide (1mg/ml) (Serva)

  - incubate reaction mix for 15`at 55°C
  - place on ice
  - add 1.2 ml of loading buffer
  - run gel in 1X MEN at ~ 60 V

A.9 Non-denaturing polyacrylamide gel electrophoresis (PAGE)

- 5X TBE (1L)

0.45 M Tris base (Roth)	54 g
0.45 M boric acid (Sigma)	27.5 g
25 mM EDTA (MP Biomedicals), pH 8	20ml of 0.5 M (v/v)

- 12% polyacrylamide gel (10 ml)

  2 ml 5X (v/v) TBE
  4 ml 30% (v/v) acrylamide (19:1 acrylamide:bis acrylamide) (Roth)
  4 ml water
  80 µl 10% ammonium persulfate (Sigma)
  6 µl TEMED (Sigma)

  - mix and pour immediately, polymerize for at least 30`

- non-denaturing gel loading buffer

  50% (w/v) sucrose (Riedel-de Haen)
  0.25 % (w/v) bromophenol blue (Sigma-Aldrich)

- RNA sample preparation

  2 µl RNA sample
  6 µl non-denaturing gel loading buffer
  12 µl nuclease-free water

A.9 (continued)

- load 2 µl of the RNA sample
- run in 1X TBE at 200 – 250 V
- stain 15 minutes in 1 mg/ml ethidium bromide (Serva)

A.10 Tricaine methanesulfonate (MS 222) stock solution for anesthetising embryos

400 mg tricaine powder (Sigma-Aldrich)
97.9 ml deionised water
2.1 ml 1M Tris base (Roth)

- adjust pH to ~7; store this 20X solution in the freezer
- for anesthetising embryos dilute 20 times with embryo test medium

A.11 Fluorescein isothiocyanate dextran solution

3% (w/v) in 0.25M KCl (Merck)

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VERSICHERUNG GEMÄSS § 5A

der Promotionsordnung der Fakultät Mathematik und Naturwissenschaften an der Technischen Universität Dresden

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

ERKLÄRUNG GEMÄSS § 5B

Die vorliegende Arbeit wurde am UFZ – Umweltforschungszentrum Leipzig-Halle GmbH unter Betreuung von Dr. Stefan Scholz (Department für Zelltoxikologie, UFZ Leipzig) und unter Betreuung von Prof. Dr. Günter Vollmer (Institut für Zoologie, Professur für Molekulare Zellphysiologie und Endokrinologie, Technische Universität Dresden) angefertigt.

Leipzig, den 30.09.2006

……

(Unterschrift Doris M. Völker)

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