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Application of rainbow trout hepatocyte cultures to study gene expression due to estrogenic compounds

Anja Schreer

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Anja Schreer, geb. am 12.02.1975 in Pritzwalk,

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Gutachter: Prof. Dr. Günter Vollmer, TU Dresden

Dr. Kristin Schirmer, UFZ Leipzig

Prof. Dr. Werner Kloas, IGB Berlin/HU Berlin

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Department Zelltoxikologie

unter Betreuung von Dr. Kristin Schirmer

und Betreuung von Prof. Dr. Günter Vollmer,

Institut für Zoologie,

Professur für Molekulare Zellphysiologie und Endokrinologie,

Technische Universität Dresden

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This thesis is dedicated to my grandmother Lore Kurth.

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Abbreviations

BSA	Bovine Serum Albumin
bp	base pair
CA	clofibric acid
cDNA	complementary deoxyribonucleic acid
CF	5-carboxyfluorescein
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
CT	threshold cycle
CYP	Cytochrome P450
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dd-rt-PCR	differential display reverse transcriptase polymerase chain reaction
DDT	p,p'-Dichlorodiphenyl-trichloroethane (pesticide)
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
E_2	17β-estradiol
EC_{50}	effect concentration where 50% of the measured effect is detected
EDTA	Ethylenediaminetetraacetic acid
EE_2	17α-ethinylestradiol
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
FBS	Fetal bovine serum
FITC	Fluoresceinisothiocyanat
GTH	gonadotropic hormone
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HSP	heat shock protein
K_{ow}	octanol/water partition coefficient
L-15	Leibovitz`s L-15 medium
LB	Luria Bertani (broth)
LOWESS	Locally weighted regression and smoothing scatterplots
LPL	lipoprotein lipase
M199	Medium H 199
mRNA	messenger ribonucleic acid
MS 222	Ethyl 3-aminobenzoate methanesulfonic acid salt
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
PBS	Phosphate-buffered saline
PCP	Pentachlorophenol
PCR	polymerase chain reaction
PMT	photomultiplier tube
PPAR	peroxisome proliferator activated receptor
qPCR	quantitative real-time reverse transcriptase polymerase chain reaction
RFU	relative fluorescence units
RNA	ribonucleic acid
RTL-W1	rainbow trout liver cell line (Lee *et al*, 1993)
rt-PCR	reverse transcriptase polymerase chain reaction
RXR	retinoic X receptor
SDS	Sodium dodecyl sulfate
SSC	Saline-Sodium Citrate-Buffer
ssDNA	single stranded DNA
TAE	Tris Acetate EDTA Buffer
TBE	Tris Borate EDTA Buffer
U	units
v/v	volume/volume
VEP	vitelline envelope protein
vol	volumetric content
VTG	vitellogenin
w/v	weight/volume

Summary

Our environment is increasingly polluted by a number of chemicals derived from the chemical and pharmaceutical industry. Many of these so called ecotoxicants are only little degraded in sewage treatment plants and therefore persistent in the environment. Thus, they are able to accumulate in the environment and constitute a constant source for exposure of organisms, which may be prone to adverse biological effects. Inasmuch as any ecotoxicant interacts with an organism by entering a cell, changes in cell structure and function comprise a sensitive means by which to explore sublethal ecotoxicant effects. Changes to cellular function can be reflected by changes in the level of gene expression, either by direct interaction with transcriptional regulation or as part of a cellular response to chemical stress. Thus, the expression of responsive genes after exposure to individual substances can be used to identify potentially hazardous effects and regulatory mechanisms. If such effects or mechanisms are known, complex, environmental samples which may contain unknown constituents can subsequently be screened for their ability to elicit the same effects and help direct chemical analysis.

Based on this background, this thesis aimed at establishing primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes as an experimental *in vitro* system to analyse and better understand multiple gene expression in this important model of aquatic (eco)toxicology. Rainbow trout hepatocytes were explored for the simultaneous expression of multiple genes with the final goal of developing a user-defined cDNA-array, focussing in particular on the detection of estrogenic effects. For this purpose, primary rainbow trout hepatocyte cultures were characterized first with regard to the maintenance of cell viability and metabolic activity throughout the time of culturing. A non-invasive cell viability assay consisting of a combination of two fluorescent dyes (alamarBlue™ and CFDA-AM) was adapted to primary rainbow trout hepatocytes and validated for its applicability prior to gene expression analysis. The application of this viability assay proved very useful in terms of monitoring the intactness of the cultures and assessing potential impacts of the investigated ecotoxicants on hepatocyte viability.

Upon establishment of the non-invasive fluorescent assays for controlling cell viability in gene expression studies, gene responses upon exposure to the natural hormone 17β-estradiol and the synthetic hormone 17α-ethinylestradiol were investigated by means of quantitative real-time rt-PCR. Eight genes were explored with regard to their response to estrogens. These genes were β-actin, vitellogenin, vitelline envelope protein α, β, γ, CYP3A27, transferrin and serum albumin. Among these, β-actin was validated as an appropriate house-keeping gene while transferrin and serum albumin showed a moderate regulation either as a response to culturing or the exposure to the estrogens. The genes coding for the vitelline envelope proteins α, β, γ proved as sensitive estrogen-regulated genes but vitellogenin overall appeared as a more robust marker of an estrogenic response on the gene expression level. As for the metabolically

relevant cytochrome CYP3A27, an estrogen receptor-dependent control was identified for the first time. These investigations confirmed the suitability of the rainbow trout hepatocytes in studying estrogenic effects on the level of gene expression.

The well characterized test system regarding estrogenic responses was, in a next step, used for determining the effects of the pharmaceutical clofibric acid. This substance is of environmental concern because of its persistency to sewage treatment and its resulting release into the aquatic environment. Potential alterations in mRNA abundance of six genes (β-actin, lipoprotein lipase, vitellogenin, CYP3A27, transferrin and serum albumin) were investigated by means of real-time rt-PCR. Interestingly, no induction of lipoprotein lipase was found upon exposure to clofibric acid although lipoprotein lipase is one key enzyme in fatty acid catabolism and one target of the fibrates to which clofibric acid belongs. Additionally, a clofibric acid dependent vitellogenin response in both sexes was detected for the first time. As well, a slight mitochondrial proliferative effect was detected based on increased rates of conversion of alamarBlue, which was applied together with CFDA-AM to the cells prior to mRNA extraction.

The genes studied in their regulation by real-time rt-PCR upon exposure of hepatocytes to 17β-estradiol, 17α-ethinylestradiol and clofibric acid, and many more genes identified by literature search, were finally applied to design a user-defined hepatocyte-specific rainbow trout cDNA-array. Two sets of arrays with varying numbers of genes were developed and evaluated with regard to gene expression. Two genes were found for the first time to be under estrogenic control in fish, namely the heat shock protein HSP 70 and the cytochrome CYP11B. Another gene, cyclooxygenase 2, was found to be regulated by 17α-ethinylestradiol in rainbow trout hepatocytes for the first time. Taken together, the user-defined array allows to compare the expression of many genes in parallel but at the same time focus on the genes of interest. It represents an efficient method for determining fingerprints of gene expression elicited by ecotoxicants in the liver of rainbow trout.

In summary, this thesis established primary rainbow trout hepatocytes as a suitable tool for investigating endocrine disrupting effects on the gene expression level *in vitro*. Furthermore, the combined alamarBlue/CFDA-AM assay proved to be very useful for the determination of acutely toxic concentrations of the substance tested but also for determining subtle effects e.g. to mitochondria. It provides an easy and valuable tool for the fast pre-screening of ecotoxicants. A variety of genes was investigated in detail by means of quantitative PCR and, together with other selected genes, applied in examinations using a user-defined cDNA-array focussed on special liver functions. In the future, this targeted array, together with quantitative real-time rt-PCR for validation, should be useful to explore multiple gene expression due to a variety of ecotoxicants alone or in complex mixtures and thus increase our understanding of subtle, adverse effects in fish.

Zusammenfassung

Unsere Umwelt wird zunehmend mit einer Vielzahl von Chemikalien aus der chemischen und pharmazeutischen Industrie belastet. Viele dieser Umweltschadstoffe werden in Kläranlagen unzureichend abgebaut und liegen deshalb persistent in der Umwelt vor. Somit sind sie in der Lage, in der Umwelt zu akkumulieren und als ständige Expositionsquelle für lebende Organismen zu fungieren, in denen sie schädliche biologische Effekte auslösen können. Ein Umweltschadstoff interagiert mit einem Organismus sobald dieser in die Zelle eindringt; die Folgen sind Veränderungen in der Zellstruktur und –funktion. Diese Auswirkungen auf Zellebene können genutzt werden, um subletale Wirkungen der Umweltgifte zu untersuchen. Zelluläre Funktionstörungen können von Änderungen in der Genexpression begleitet werden, entweder durch direkte Beeinflussung der transkriptionellen Regulation oder als Teil der zellulären Streßantwort. Somit kann nach der Exposition mit bestimmten Substanzen die Expression entsprechend sensitiver Gene für die Identifizierung potenziell gefährlicher Wirkungen und deren Regulationsmechanismen genutzt werden. Wenn solche Effekte oder auch Mechanismen bekannt sind, können nachfolgend komplexe Umweltproben unbekannter Zusammensetzung auf die gleiche Weise untersucht werden und somit der direkten chemischen Analyse bei der Suche nach den aktiven Substanzen behilflich sein.

Vor diesem Hintergrund war es das Ziel dieser Dissertation, primäre Hepatozytenkulturen aus der Regenbogenforelle (*Oncorhynchus mykiss*) als experimentelles *in vitro* System für die Analyse und zum besseren Verständnis der Genregulation in diesem bedeutenden Modell der aquatischen (Öko)Toxikologie zu etablieren. Hepatozyten der Regenbogenforelle wurden erstmalig mit einem benutzerdefinierten cDNA-Array mit dem Ziel untersucht, östrogene Wirkungen simultan an einer Vielzahl von Genen zu detektieren. Zu diesem Zweck wurden primäre Hepatozytenkulturen der Regenbogenforelle zuerst hinsichtlich der Erhaltung der Zellvitalität und der metabolischen Aktivität über den Zeitraum der Kultivierung charakterisiert. Ein nicht-invasiver Zellvitalitätstest, bestehend aus einer Kombination von zwei Fluoreszenzfarbstoffen (AlamarBlue™ and CFDA-AM), wurde an die Hepatozytenkultur angepaßt und hinsichtlich seiner Anwendbarkeit direkt vor Genexpressionsanalysen überprüft. Der Einsatz dieses Vitalitätstests beim Überwachen der Zellkultur sowie beim Abschätzen potenzieller Einwirkungen der untersuchten Umweltschadstoffe auf die Hepatozytenvitalität wurde als äußerst nützlich eingestuft.

Nach der Etablierung des nicht-invasiven Fluoreszenz-Tests zur Kontrolle der Zellvitalität vor Genexpressionsstudien wurden nach Einwirkung des natürlichen Hormons 17β-Östradiol und des synthetischen Hormons 17α-Ethinylöstradiol, Veränderungen in der Expression von acht verschiedenen Genen mittels real-time rt-PCR untersucht. Dazu gehörten β-Actin, Vitellogenin, die Gene der Eihüllproteine (Vitelline Envelope Protein α, β, γ), CYP3A27, Transferrin und Serumalbumin. β-Actin wurde als geeignetes Housekeepinggen bestätigt, während Transferrin

und Serumalbumin, entweder als Reaktion auf die Kultivierung oder als Antwort auf die Östrogene, eine moderate Regulation zeigten. Die Gene für die Vitelline Envelope Proteine α, β, γ wurden als sensitive, östrogenregulierte Gene ermittelt, im Vergleich dazu aber erschien Vitellogenin als zuverlässigster und stabilster Anzeiger östrogener Wirkungen auf Genexpressionsebene. Für das metabolisch relevante Cytochrom CYP3A27 wurde die östrogenrezeptorabhängige Regulation erstmals beschrieben. Diese Untersuchungen bestätigen die Hepatozytenkulturen der Regenbogenforelle als geeignetes Objekt, um östrogene Wirkungen auf Genexpressionsebene zu studieren.

Der nächste Schritt beinhaltete die Verwendung dieses gut charakterisierten Testsystems bei Untersuchungen von Effekten, die durch den Arzneimittelwirkstoff Clofibrinsäure ausgelöst werden können. Diese Substanz ist insofern umweltrelevant, als sie nahezu unverändert Kläranlagen durchläuft und somit in die aquatische Umwelt entlassen wird. Potentielle Veränderungen der mRNA Menge von sechs ausgewählten Genen (β-Actin, Lipoproteinlipase, Vitellogenin, CYP3A27, Transferrin und Serumalbumin) wurden mit Hilfe von real-time rt-PCR untersucht. Interessanterweise wurde keine Induktion der Lipoproteinlipase gefunden, obwohl diese eines der Schlüsselenzyme im Fettsäuremetabolismus und somit in der therapeutischen Wirkung der Clofibrinsäure ist. Außerdem wurde zum ersten Mal eine clofibrinsäureabhängige Vitellogeninantwort in beiden Geschlechtern detektiert. Desweiteren wurde eine schwache Proliferation der Mitochondrien aufgrund von gesteigerter AlamarBlue-Umsetzung in den fluoreszierenden Farbstoff Resorufin entdeckt.

Die Gene, welche mittels real-time rt-PCR nach 17β-Östradiol, 17α-Ethinylöstradiol und Clofibrinsäure auf ihre Regulation hin untersucht wurden, sowie viele weitere, die durch Literaturrecherche ermittelt werden konnten, wurden für einen benutzerdefinierten, hepatozytenspezifischen cDNA-Array der Regenbogenforelle verwendet. Es wurden zwei Array-Sätze mit unterschiedlicher Genzahl entwickelt und angewandt. Zwei Gene, das Hitzeschockprotein HSP 70 und das Cytochrom CYP11B, wurden zum ersten Mal als östrogenreguliert in Fischhepatozyten entdeckt. Ein weiteres Gen, die Cyclooxygenase 2, wurde in Regenbogenforellenhepatozyten erstmalig als reguliert durch 17α-Ethinylöstradiol beschrieben. Aufgrund der gleichzeitigen Untersuchung vieler Gene ist der benutzerdefinierte cDNA-Array eine effiziente Methode, sogenannte Fingerabdrücke der Genexpression, ausgelöst durch bestimmte Substanzen, in der Leber der Regenbogenforelle zu erstellen.

Zusammenfassend kann festgestellt werden, daß innerhalb der vorliegenden Dissertation die Regenbogenforellenhepatozyten als geeignetes *in vitro* Modell zur Untersuchung von hormonellen Störungen auf Genexpressionsebene etabliert wurde. Desweiteren wurde der kombinierte AlamarBlue/CFDA-AM Test im Hinblick auf die Abschätzung akut toxischer Wirkungen der zu testenden Substanz als sehr nützlich validiert, konnte aber auch bei der Bestimmung von Effekten auf z.B. die Mitochondrien eingesetzt werden. Dieser Test bietet sich als einfache und nützliche Methode für eine erste, schnelle Einordnung von Umweltschadstoffen

an. Eine Reihe von Genen wurde eingehend mittels real-time rt-PCR untersucht und zusammen mit anderen ausgewählten Genen unter Verwendung eines benutzerdefinierten cDNA-Array auf spezielle Leberfunktionen untersucht. Dieser gezielt ausgerichtete Array kann bei der Untersuchung der Expression von einem Gensortiment nach Exposition mit einer Vielzahl von Umweltschadstoffen entweder einzeln oder in Kombination zusammen mit der real-time rt-PCR als Validierungsmethode sehr nützlich sein. Er trägt somit zur Gewinnung von Kenntnissen über schädliche Wirkungen auf den Organismus Fisch bei.

Chapter 1

General Introduction

There have been growing concerns in recent years about chemicals in the environment interfering with hormone systems. An increasing number of xenobiotic environmental contaminants (ecotoxicants) and natural compounds are suspected of modulating endocrine regulation in humans as well as animals. A major sink of these contaminants is the aquatic environment, with sewage being a major contributor. Consequently, aquatic organisms are among those most directly affected by endocrine modulators that can ultimately lead to endocrine disruption.

This chapter provides the basal knowledge about fish endocrinology and a definition of endocrine disruptors. Furthermore, a short introduction into primary hepatocytes from rainbow trout as model system is given, and the relatively new quantitative real-time rt-PCR and cDNA-array technique are described. Finally, the aim and a brief outline of this thesis are presented.

1.1 The endocrine system

The following description of the endocrine system focusses on the pathway of the sexual hormones in fish (hypothalamus-pituitary-gonadal-axis). In general, only neural information from the central nervous system is able to activate a complete hormonal cascade. Every hormonal cascade starts in the hypothalamus (Figure 1.1). Gonadotropin-releasing-hormones (GRH) are secreted from the neuro-secretory cells of the hypothalamus following the neural stimulus. The GRHs are transported via the portal system to the pituitary gland, where they stimulate the secretion of the gonadotropic hormones (GTHs). The main function of the GTHs is to enter the gonads and to induce the expression and secretion of the sexual hormones (androgens and estrogens). The sexual hormones are bound to steroid binding proteins and carried via the blood system to the target organs. The major target organ for estrogens is the liver, where they induce vitellogenesis, while androgens act in the testis and cause the production of sperm. In addition to its vitellogenic function, the liver is also the major site of catabolism of the steroid hormones. This complete cascade is regulated by a feedback mechanism determined by the concentration of the sexual hormones and GTHs.

The two gonadotropic hormones have different nomenclature in mammals and fish. The follicle stimulating hormone (FSH) in mammals is called GTH I in fish and the luteinizing hormon is entitled GTH II (Kawauchi *et al*, 1989). GTH I is responsible for the development of the oocyte and vitellogenesis or spermatogenesis, while GTH II triggers the changes in steroidogenesis for the final maturation of the gamete. In the early development of the oocyte, GTH I is present at increased concentrations in the blood stream. It binds to its receptor localized at the outer theca layer of the follicle of the developing oocyte, and induces the synthesis of testosterone. The inner granulosa layer converts testosterone to estradiol via aromatization (Kagawa, 1985, Nagahama, 1987, Adachi *et al*, 1990). Both steroid hormones are released into the blood stream. Circulating estradiol binds to hepatic receptors and subsequently stimulates the expression and secretion of the egg yolk precursor protein vitellogenin (Nagahama, 1983), in addition to the development of the secondary sexual characteristics. Interestingly, the liver of male fish has the necessary receptors for binding estradiol, although estradiol is not normally present in male. Vitellogenin is a glycosylated lipoprotein that is produced in the liver of oviparous vertebrates. It is transported by the blood to the oocyte where it is incorporated by the vitellogenin receptor for energy reserves (Dolphin *et al*, 1971, Tyler *et al*, 1990). During the maturation of the oocyte, the concentration of GTH I decreases and the GTH II increases (Kawauchi *et al*, 1989, Swanson *et al*, 1991). The receptor for GTH II is mainly localized at the granulosa layer of the follicle. The receptor-ligand-complex initiates the synthesis and secretion of progestogen. This hormone is primarily responsible for the maturation of the gametes and the stimulation of ovulation (Swanson *et al*, 1991).

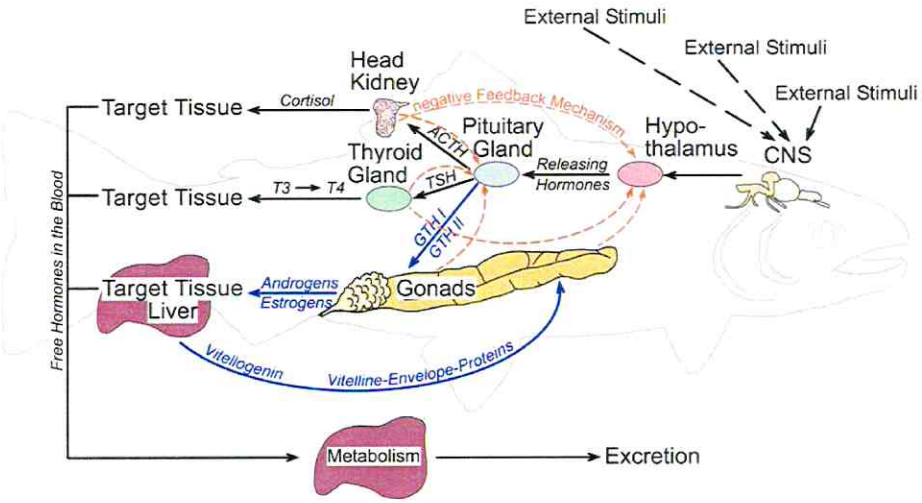

Figure 1.1 Hormonal system in fish. The cascade of the sexual hormones also called hypothalamus-pituitary-gonadal-axis is depicted in blue. For details see text above.

1.2 Endocrine disruption

A definition for endocrine disruptors can be found in (Dankwardt, 1998):

"An environmental endocrine or hormone disruptor may be defined as an exogeneous agent that interferes with the synthesis, secretion, transport, binding, acting or elimination of natural hormones in the body, that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior."

The first comprehensive work expressing concern about the impact of man-made chemicals on the environment was published by Rachel Carson (1962) in her book "The silent spring". Decades later another book "Our stolen future" from Colborn *et al.* (1996), led to increased public attention to anthropogenic substances released into the environment and causing endocrine disruption. The accumulation of adverse effects on the process of reproduction in wildlife (e.g. behavioral abnormalities, malfunctioning gonads in the offspring) (reviewed in Guillette & Gunderson, 2001) led to expanded research interest in the chemicals and the mechanisms eliciting these effects. The most famous example of endocrine disruption occurring in wildlife is the fate of the alligator population in Lake Apopka in Florida, USA (Semenza *et al*, 1997). About 10 years after an accident at a local chemical company, the consequences, namely disruptions in the internal and external reproductive organs of the F1 generation, were recognized. This implies that endocrine disrupters are able to act in the exposed organism or in the gametes or embryos. The first steps of ontogenesis are completely controlled by hormones, particularly by the concentration of estradiol. An external impact on this sensitive system may lead to devastating malformations of the embryo.

The main concern about endocrine disruptors has been that environmental pollutants mimic endogenous estrogens and exert direct effects via the estrogen receptor (ER). This has led to a substantial research effort on ER-mediated responses at both the transcriptional and protein level (e.g. Denslow *et al*, 2001, Arukwe *et al*, 1997). Well known response endpoints on both levels are, among others, the induction of the vitelline envelope proteins and vitellogenin. Particularly, the induction of vitellogenin protein and/or mRNA is today an accepted and well recognized biomarker for exposure to estrogen or "estrogen-like" compounds.

1.3 Rainbow trout hepatocytes as model system

Rainbow trout (*Oncorhynchus mykiss*) belongs to the taxon *Teleostei* and the family *Salmonidae*. The natural habitat of this species is the cold waters in Northern America, but it has been introduced throughout the world. Two types of rainbow trout populate the world, the freshwater river trout and the euryhaline trout. In this study the freshwater rainbow trout was used as model organism.

Rainbow trout are very sensitive to several environmental factors such as pH, osmolarity, and oxygen content of the water. Due to this, rainbow trout is a popular model for investigating the impact of environmental factors. In addition, the increasing number of sequenced genes makes the rainbow trout attractive for studies looking at impacts on the molecular level. The liver as the major metabolic site and the target organ for estrogens is of special importance for examining potential harmful xenobiotics. Because of this, primary fish hepatocyte cultures are indispensable tools for advancing the understanding of fish liver physiology and toxicology (Pesonen & Andersson, 1997; Segner, 1998). One important advantage over the use of whole fish is that they allow the role of selected parameters to be investigated in a defined culture system. This is particularly useful for studying gene expression where subtle changes may be important but difficult to detect if the experimental system is less defined.

A brief history of the isolation and culturing of primary fish hepatocytes

Berry and Friend (1969) established the two step perfusion of mammalian livers. Seglen published in the early 1970s a series about the enzymatic requirements, the effect of calcium, and the effects of ions and chelators on tissue dispersion (Seglen, 1972; Seglen, 1973b; Seglen, 1973a). These methods were modified for fish in the 1980s (e.g. Klaunig, 1984; Klaunig *et al*, 1985; Andersson & Förlin, 1985; Mommsen & Lazier, 1986). There are at least two possible methods to culture primary hepatocytes. Hepatocytes can be viable in a shaking suspension culture, or conversely, an attached monolayer can be established. For the latter, various coating substances have been examined such as collagen (Kocal *et al*, 1988), skin extract (Lee & Dasmahapatra, 1993) and an extracellular matrix (Lipsky *et al*, 1986). For maintaining the vital functions of the liver cells, an appropriate medium has to be provided. Almost all types of common media (L-15, Medium 199, Minimum Essential Medium, William`s Medium E, Hank`s buffered medium) are used with a lot of different supplements such as fetal bovine serum (FBS) in various concentrations (Klaunig *et al*, 1985), HEPES, Antibiotica, Antimycotica or nutrients (Blair *et al*, 1990; Anderson *et al*, 1996). In this thesis, monolayers attached to an extracellular matrix (Matrigel), and cultured in L-15 or Medium M199 without FBS, were used.

1.4 New methods in ecotoxicology

In this thesis, two new techniques from the field of molecular biology have been applied to ecotoxicological questions. These techniques, quantitative real-time reverse transcription polymerase chain reaction (qPCR) and cDNA-arrays, are briefly introduced in the following.

Quantitative real-time reverse transcription polymerase chain reaction (qPCR)

The qPCR is a relatively new technology that emerged in the 1990s (Higuchi *et al*, 1993; Heid *et al*, 1996). It allows the increase of the quantity of DNA to be observed during the PCR amplification procedure. The use of a fluorescent dye(s) allows the observation and quantification of the amplified DNA fragments in real time. The following brief explanation focusses on the use of the fluorescent dye SYBRgreen I. SYBRgreen I is self quenching when freely present. As soon as double helices of the DNA fragments are generated, the SYBRgreen I intercalates, and its fluorescence can be measured. The melting curve derived at the end of the DNA amplification cycles for each PCR reaction gives information about the size and the amount of the amplified fragments. For this reason, the generation of a melting curve can substitute for agarose gels. Thus a rapid and sensitive method for quantifying DNA fragments is available.

cDNA-arrays

Microarrays were developed by Schena and colleagues in 1995. Since this technique was introduced, it has been applied to many different fields. At first it was used as a diagnostic application in human medicine. In the last few years a rising number of researchers discovered the potential of this technique in molecular ecotoxicology. The microarray is increasingly used as screening tool for genes that are regulated by a certain compound or environmental sample (Afshari *et al*, 1999; Pennie, 2000), and this may also be useful for investigations focussing on the mechanistic understanding of regulatory processes (Richert *et al*, 2003).

Working principle of a cDNA-array: The working principle of a microarray is based on the hybridization of dye labelled cDNAs to their corresponding fragment immobilized on the slide surface (Figure 1.2) (Xiang & Chen, 2000; Larkin *et al*, 2003b). There are two types of microarrays, one is based on oligonucleotides and the other on cDNA fragments immobilized on a surface. This description focusses on the latter type. The production of a cDNA-array involves the spotting of amplified cDNA fragments of interest onto a pre-treated glass slide (e.g. poly-L-lysin, epoxy or aminosilane), and the immobilization of only single stranded DNA. A spotted array can be stored for several months in a dry, dark place.

Samples are prepared, for investigation using a microarray, by isolating RNA from both a treated and a control sample. Subsequently, these RNA samples are labelled with different

fluorescent dyes either directly during the cDNA synthesis by incorporation of dye-coupled deoxynucleotide-triphosphates, or indirectly by coupling the dyes on aminoallyl tails which are part of modified deoxynucleotide-triphosphates in the cDNA. Treated samples as well as control samples are labelled with different dyes. Equal amounts of both labelled cDNAs are combined and added to the array for hybridization. After several hours, unbound cDNA is removed by washing steps and the fluorescent intensity of the dyes at each spot is scanned. By comparing the intensity of the dyes, information is given about the relative ratio of mRNA abundance in the control and treated sample. The advantage of this simple technique is the possibility to monitor high numbers of genes in parallel in a short time.

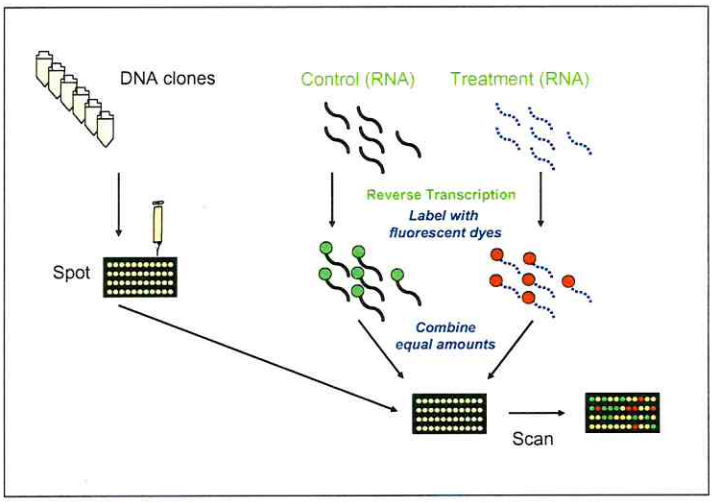

Figure 1.2 Systematical scheme of a cDNA-array and its mode of operation, for detailed explanation see text.

The application of primary rainbow trout hepatocyte cultures for studying ecotoxicological effects using sophisticated methods such as cDNA-arrays and quantitative real-time rtPCR opens a new research area called toxicogenomics. The possibility to analyse expression of numerous genes in parallel following exposure to environmental pollutants will offer insights into the molecular mechanisms of toxicity or chronic effects, providing sensitive new methodologies for environmental risk assessment.

1.5 Aim and structure of this thesis

The major goals of the present study were to characterize the effects of three environmental contaminants on the expression of several genes in primary rainbow trout hepatocytes and to introduce new molecular techniques to the field of ecotoxicology. This work is presented, following this introductory Chapter 1, in three main Chapters.

Firstly, a complete description of the materials and methods used in this thesis is provided in **Chapter 2**.

Chapter 3 includes all experimental results and their discussion in four subchapters:

In **Chapter 3.1** a non-invasive cell viability assay based on two fluorescent dyes was adapted to the hepatocyte culture and examined for its potential applicability to hepatocytes just prior to RNA isolation and gene expression without impacting on the latter.

The impact of 17β-estradiol and 17α-ethinylestradiol on the cell viability and the expression of eight genes in a dose- and time-dependent manner using qPCR was investigated in **Chapter 3.2**. The genes were those coding for β-actin, vitellogenin, vitelline envelope protein α, β, γ, CYP3A27, transferrin and serum albumin.

As a representative of human pharmaceuticals increasingly detected in the aquatic environment but unknown so far with regard to potential impacts on non-target species, such as fish, the effects of clofibric acid on cell viability and the transcription of six selected genes are shown in **Chapter 3.3**. The genes were β-actin, lipoprotein lipase, vitellogenin, CYP3A27, transferrin and serum albumin.

In order to further improve the efficiency of exploring the expression of a number of genes in the primary rainbow trout hepatocyte model, **Chapter 3.4** aimed at developing a user-defined rainbow trout cDNA-array for detecting estrogenic effects of environmental toxicants on the transcriptional level.

The final **Chapter 4** contains the comprehensive conclusions of this work and ideas for further research on this topic.

Chapter 2

Materials and Methods

2.1 Definitions

An independent experiment was defined as the exposure of hepatocytes from a single fish. For this reason, comparisons between independent experiments mean comparisons between individuals. The number of independent experiments = individual fish were named N. Each 24 well plate within an independent experiment contained three replicate wells of blank control with non-treated cells, three replicate wells of solvent control and three replicate wells of each concentration of the tested compound. For the cell viability assay, these triplicate wells were n = 3. In contrast to that, the cells of these three replicate wells were pooled for RNA isolations and subsequent gene expression examinations. Here, n represents the measurement of three replicate wells of the 96 well plate in the real-time rt-PCR (qPCR).

All chemicals were purchased from MERCK, Germany if not stated otherwise.

2.2 Establishment of primary hepatocyte cultures

Primary hepatocytes were isolated from rainbow trout (*Oncorhynchus mykiss*) that were kept in 540 L tanks with a permanent flow of fresh, air saturated water under a 12 h dark/light cycle. Immature rainbow trout weighing 150-250 g were used for hepatocyte isolation using a two-step collagenase perfusion technique as described for mammals (Seglen, 1972) with modifications for fish (Maitre *et al*, 1986). The fish were left without feeding for one day before hepatocyte isolation. Each trout was anaesthetized with 0.8 g MS 222 (Ethyl 3-aminobenzoate methanesulfonic acid salt from Aldrich, USA) in 10 L water for 10 min. The heart should be beating until successful cannulation and perfusion because a pumping heart prevents blood from clumping and coagulating. The narcoticized fish was carefully wiped with ethanol and placed on a surgical board in a laminar flow hood, with its left side upwards. A ventral cut from the anus to the gills was used to open the fish. With three further incisions a square was cut out. The portal vein was cannulated with a blood vacutainer, size 23G ¾ (Becton Dickenson, USA) approximately 1 cm from the liver. The cannula was fixed with a surgical thread. Perfusion was initiated using pre-perfusion buffer (169 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.63 mM Na_2HPO_4, 26 mM $NaHCO_3$, 5.5 mM Glucose (Sigma, Germany), 25 mM HEPES (SERVA, Germany), 1.1 mM EGTA (Sigma, Germany)) for 10 min with a flow rate of 5 mL/min. The liver

blanched immediately. During this period the heart was removed to avoid pressure in the liver. The perfusion was continued for 8-12 min with a second pre-perfusion buffer which contained 4000 U collagenase Type 2 (Worthington, USA) and 0.2 % [w/v] $CaCl_2$ instead of EGTA. During this perfusion step the liver lost its shape and elasticity. Afterwards the liver was perfused for 3 min with washing buffer, consisting of medium M199 (complemented with 3.7 mM HEPES, 4.2 mM $NaHCO_3$, 0.9 mM $CaCl_2$) without glutamine and antibiotics but with 1 % [w/v] bovine serum albumin (Sigma, Germany). After perfusion, the liver was excised, sieved and washed following the description outlined in Oppen-Berntsen *et al.* (1992) yielding a single cell suspension.

The resulting hepatocyte suspension was kept in serum- and phenol red-free L-15 medium (GIBCO, USA) supplemented with 1 % [w/v] penicillin/streptomycin solution (10,000 units penicillin, 10 mg/ml streptomycin in 0.9 % [w/v] NaCl, SIGMA, Germany). In a few experiments (see Chapter 3.1), cells were, in addition to L-15, prepared in M199 (ICN, USA), supplemented with 3.7 mM HEPES, 4.2 mM $NaHCO_3$, 0.9 mM $CaCl_2$, 2 mM L-Glutamine (Sigma, Germany) and 1 % [v/v] penicillin/streptomycin solution (10,000 units penicillin, 10 mg/ml streptomycin in 0.9 % [w/v] NaCl, SIGMA, Germany). Cells possessed more than 90 % cell viability as estimated by Trypan Blue exclusion. In brief, 200 µl of Trypan Blue (0.5 % [w/v] solution, SERVA, Gemany) was diluted 4 times in an aliquot of the cell suspension. Subsequently, the solution was mixed and cells were counted by means of a hemacytometer, dead cells appeared blue and live cells appeared translucent. Cells were seeded at a densitiy of $1x10^6$ in 24-well plates (Falcon 3847 Primaria, Becton Dickenson and Company, USA) in a total volume per well of 1 mL. Prior to use, the 24-well plates were coated with Matrigel (BD Biosciences, USA) by incubating the uncovered plates for approximately 2 h in a laminar flow hood containing 200 µL in M199 diluted Matrigel solution (final concentration 0.1 mg/mL) and a subsequent washing step with M199. Cells were incubated at 19°C in a normal atmosphere of air in the dark.

2.3 Culture of the rainbow trout liver cell line, RTL-W1

The fish liver cell line was used in a few experiments in Chapter 3.1 as an additional model for investigating gene expression patterns, upon performance of the cell viability assays, using differential display rtPCR (see below). As well the ability of the cell viability assay to detect toxicity by pentachlorophenol (PCP) was additionally observed by RTL-W1. RTL-W1 (Lee *et al*, 1993) is an immortalized cell line from a rainbow trout liver. It was cultured in L-15 medium supplemented with 5% [v/v] FBS (Biochrom, Germany) and 1 % [v/v] penicillin/streptomycin (10,000 units penicillin, 10 mg/ml streptomycin in 0.9 % [w/v] NaCl, SIGMA, Germany) in an atmosphere of air in the dark at 19°C. Culture flasks (75 cm^2, Nalge Nunc, Belgium) containing confluent monolayer cultures of RTL-W1 were exposed to the mixture of fluorescent indicator dyes as described below. After 30 min of incubation, the cells were scraped from the flask, washed with PBS buffer and frozen at 20°C in RNAlater (Ambion, UK) until RNA isolation and

application to differential display rt-PCR. For exposure to PCP, RTL-W1 cells were plated at a density of 75,000 cells/well in 48 well multi plates.

2.4 Exposure of cells

Cells were exposed to two different solvent contents over the duration of this dissertation (≤ 1 % v/v in Chapter 3.1, 0.5 % v/v in Chapter 3.2 and ≤ 0.5 % v/v in Chapter 3.3) (ethanol, MERCK-Schuchardt, Germany). Varying concentrations of 17β-estradiol (E_2, Sigma, Germany), 17α-ethinylestradiol (EE_2, Sigma, Germany), clofibric acid (CA, ICN Biomedicals, USA) (for chemical structures see appendix Figure A1) and, where indicated, the estrogen receptor antagonist ICI 182,780 (Tocris, USA), all were dissolved in ethanol. The first dosing was done 18 h after seeding the hepatocytes and was initiated with a half-media change. At this time hepatocytes had attached and started to form aggregates. Every day thereafter, a half-media change was performed over up to four days of exposure. The appropriate amount of the investigated compound was re-added in order to maintain a consistent exposure concentration. Upon termination of exposure, cell cultures were processed for both cell viability assessment and mRNA quantification.

The initially selected E_2 (10^{-8} M – 10^{-5} M) and EE_2 (10^{-9} M – 10^{-6} M) concentrations were chosen in order to achieve maximal VTG mRNA induction levels. The chosen range was in accordance with previous studies, where a maximal VTG induction on the transcriptional as well as on the translational level was found. For instance, Vaillant *et al.* (1988) tested the mRNA induction of VTG with dot hybridization in primary hepatocytes from male rainbow trout at 10^{-9} M – 10^{-5} M E_2. Furthermore, Navas & Segner (2000) used 10^{-9} M – 10^{-5} M E_2 for VTG protein measurements. In many other studies a positive induction control was performed at 10^{-6}M E_2 (e.g. Madigou *et al*, 2001; Anderson *et al*, 1996). Based on the high responsiveness of the hepatocytes with regard to VTG induction in the course of this thesis, concentrations of E_2 of 10^{-12} to 10^{-9} M were also used in one instance (Chapter 3.2).

For CA (Chapter 3.3), the concentration range was chosen, on one hand, so as to include the rather low concentrations that were detected in Berlins surface- and groundwater and sewage treatment effluents (Stan & Heberer, 1997). On the other hand, for allowing potential thus far unknown effects to be discovered, relatively high concentrations were included also.

The concentrations of the estrogen receptor antagonist ICI 182,780 were selected to be comparable to the concentrations of the substance tested.

2.5 Assessment of cell viability using fluorescent indicator dyes

2.5.1 Procedure

Cell viability was assessed using a combination of two fluorescent indicator dyes, alamarBlue™ (BioSource, Germany) and CFDA-AM (Molecular Probes, USA). The procedure followed the detailed description outlined in Schirmer *et al.* (1997) except that dye solutions were prepared in double concentration (10 % [v/v] alamarBlue and 8 µM CFDA-AM) to account for the dilution in the medium remaining during the half-medium change.

2.5.2 Data analysis

Raw data were statistically evaluated for their dependencies on time or concentration using one way ANOVA followed by the Dunnett test in order to determine the substance concentration that caused a significant decrease of cell viability compared to the solvent control. P values of < 0.05 were considered to be significant. Statistical analyses were performed using GraphPad Prism Software (Version 4.0, USA). For data representation, raw data were generally converted to % of control as described by Schirmer *et al.* (1997).

2.6 VTG-ELISA

The medium was removed from cultures and stored at -20°C until use. VTG protein in the hepatocyte culture medium was determined by ELISA (Biosense, Norway) according to the instruction provided by the supplier.

2.7 Differential display (dd) rt-PCR

In order to investigate if general differences in gene expression could be observed in cells subjected to the fluorescent cell viability assays or not, a fluorescent-based modification of the Stratagene differential display protocol (RAP PCR Kit) was applied (Chapter 3.1). First strand cDNA synthesis (Reverse Tanscriptase Kit, Qiagen, Germany) of the hepatocyte mRNA was done with the same random primer(s) (1 µM final concentration) as subsequent PCR thereby the primers used in reverse transcription were unlabelled. For the samples from the RTL-W1 cell line, oligo dT primer was used for cDNA synthesis. The random primers A3 (5'-AAT CTA GAG CTC TCC TGG-3'), A2 (5'-AAT CTA GAG CTC CAG CAG-3') and A4 (5'-AATCTA GAG CTC TCC AGC-3') available from a variety suggested by Stratagene were selected. These primers were labelled with a fluorescent Texas Red tag at the 5' site (Metabion, Germany) for dd-rt-PCR. For PCR amplification, 5 µL of the cDNA template were used in 25 µL reactions containing 2.5 µL of the labelled random primer (10 µM final concentration), 3 mM $MgCl_2$ and Ready-To-Go PCR beads (Amersham Pharmacia Biotech Inc., USA). PCR conditions were as follows: 1 min at 94°C, 5 min at 36°C, 5 min at 72°C and 40 cycles of 1 min at 94°C, 2 min at 50°C and 2 min at 72°C. The dd-rt-PCR was performed in a Thermocycler (T3, Biometra, Germany). The results of

this PCR procedure were fluorescently labelled DNA fragments of various sizes. The fluorescently labelled PCR products were separated on a 6 % polyacrylamide gel prepared in 1xTBE buffer (90 mM Tris, 90 mM Boric acid, 2 mM Na_2EDTA (pH 8.0)) using the Sequi Gen GT gel apparatus (Bio-Rad, USA). PCR products were visualized using the Molecular Imager FX from BioRad (USA).

2.8 Assessment of mRNA abundance using quantitative real-time rt-PCR (qPCR)

2.8.1 Procedure

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Germany) and the concentration determined spectrophotometrically (λ = 260 nm). The quality of the RNA as well as the quantity were verified using electrophoresis on a 1 % [w/v] agarose gel in 1xTAE (40 mM Tris, 20 mM glacial acetic acid, 1 mM NA_2EDTA, pH 8.0) containing 50 ng/ml ethidium bromide. Electrophoresis was performed using 1xTAE as buffer. The used voltage varied between 80 and 100 V depending on the size of the chamber. Subsequently, the bands were visualized and documented by means of a UV-transilluminator using a video camera (GelDoc sytem from BioRad, USA). First strand cDNA synthesis was done with an oligo dT primer and Omniscript Kit (Qiagen, Germany). Real-time rt-PCR (iCycler, BioRad, USA) was used to quantify the amount of mRNA. The PCR was performed with chemicals of the Taq-PCR-Core Kit supplied by Qiagen (Germany) in 96 well plates (BioRad, USA) using the following reagents and final concentrations (see also Table 2.1): 10 nM FITC (Fluoresceinisothiocyanat, BioRad; used to adjust background fluorescence), 0.1 nM primers, 5 mM $MgCl_2$, 0.2 mM dNTPs and 1 U Taq Polymerase. The following PCR conditions were used: 3 min at 95°C and 40 cycles of 20 sec at 55°C, 20 sec at 72°C, 20 sec at 95°C ; 30 sec at 60°C. SYBRgreen I fluorescence was determined during the elongation phase. A melting curve was generated to evaluate the quality of the PCR product. For calculating the threshold cycles (CT), the threshold position was fixed at 40,000 relative fluorescence units (RFU). Samples with threshold cycles above 30 were considered below the level of detection and not included into data analysis. The inter-assay coefficient of variation was 4.1% (number of independent PCR = 15). All samples were measured in triplicates. For data normalisation, β-actin was used as the house-keeping gene.

Table 2.1 Overview of selected target genes and qPCR parameters

Gene	Comments to gene	GenBank Accession No.	Primer sequence[1] (5' – 3')	Product length (bp)	Amplification efficiency	SYBRgreen I conc.
β-actin	house-keeping gene	AJ 438158	CACCGTAAATGCTTCTAAACAG AATCTTTAATCCGCTGCTTCAC	255	96%	1x
Vitellogenin	precursor of egg yolk protein	M 27651	AACCAAGTCAGCCAGGTAATATG GAACGACAACTGGAAACTGTGT	252	88%	1x
VEPα	Vitelline envelope protein α	AF 231706	GACATTACCAGGGACAGCC CCTTGGAGAGACAACGTCC	175	97%	1x
VEPβ	Vitelline envelope protein β	AF 231707	TGGTTATTGATGTGAAGCCG GCGAACCTCAGTGTAGACAGG	200	99%	1x
VEPγ	Vitelline envelope protein γ	AF 231708	ACCTGAAGGCAACCACAATC ATGGGACCAAGCTGAACATC	214	87%	0,5x
CYP3A27	hydroxylation of testosteron and progesteron[2], 55% amino acid sequence homology to the human cytochrome CYP3A4[3]	U 96077	AGCAGGCAGATAAAGACCAGAC GTGAAGGGAAACAAAGCGAC	202	107%	1x
Transferrin	serum protein for iron transport, produced mainly in the liver	D 89083	AATGGTTAGATGGTGCGTAAAG GCAGGTTGTAGTTGTGGAGG	197	95%	1x
Serum Albumin	major serum protein with relatively low affinity to many potential ligands, produced in the liver	X 52397	AGCAAGGATCTGCTGCTCTC ACCTTGACCAGCTCTGCACT	215	87%	1x
Lipoprotein lipase	hydrolysis of trigyceride	AF358669	CTACACTGAGCAGCCCATGA GCGGATGTGGAAGTTTTTGT	220	87%	1x

[1] Primers were designed using Primer3 software and the product size confirmed by conventional rt-PCR and gel electrophoresis prior to use in the real-time rt-PCR.

[2] Buhler & Wang-Buhler (1998)

[3] Lee *et al.* (1998)

2.8.2 Data analysis

Results of the real-time rt-PCR were calculated relative to the house-keeping gene β-actin according to the normalization procedure of the *Q-Gene Core Module* (Müller *et al*, 2002). This normalization procedure takes varying PCR amplification efficiencies into account (see Table 1). In brief, threshold cycles for triplicate determinations for, respectively, the target and the reference gene (CT_{target} and CT_{ref}) were averaged. Next, the PCR amplification efficiency values for each gene were taken to the power of their respective mean CT_{target} and CT_{ref}. Based on these values, the ratio of the target gene versus the house-keeping gene was calculated to yield the mean normalized gene expression. Thus, the mean normalized gene expression is representative of the relative expression level, or mRNA abundance, of the target gene compared to the house-keeping gene. Additionally, the standard error of the mean normalized gene expression was calculated based on the differential equation of Gauss (Müller *et al*, 2002).

The normalized expression data were statistically evaluated for their dependencies on time or concentration using one way ANOVA followed by the Dunnett test. To estimate the impact of the combination of concentration and time, two way ANOVA was performed. For determination of differences between positive control and treatment with various ICI 182,780 concentrations unpaired one-tailed t-test was used. P values of < 0.05 were considered to be significant. Statistical analyses were performed using GraphPad Prism Software (Version 4.0, USA).

2.9 cDNA-array Experiments

2.9.1 Selection of genes

Based on the literature, genes were firstly selected according to common knowledge on trout liver as a target for estradiol. In addition, genes that have been implied to be involved in cross-regulating estrogenic mechanisms were identified. Other genes were those known to have a basic function in the liver, e.g. by playing a role in hexose mobilisation, stress response or xenobiotic metabolism. As well, a set of genes was included intended in use as house-keeping genes. A complete list of all spotted gene fragments is provided in the appendix.

2.9.2 Probe preparation

One part of the probes was a gift from Tom Mommsen, University of Victoria, B.C., Canada. The other part was produced by the following procedure. Total RNA from rainbow trout liver was isolated and cDNA synthesized as described above (2.7.1). The fragment of interest was amplified with specific primers and size and quality checked by agarose gel electrophoresis. The bands were cut out and purified with the QIAquick Gel Extraction kit (Qiagen, Germany). The subsequent cloning into *E.coli* was done with Topo TA cloning kit (Invitrogen, USA) according to the user manual. The transformed bacteria were plated in petri dishes containing 10 g/L

tryptone (Becton Dickenson and Company, USA), 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar (Becton Dickenson and Company, USA), 50 mg/mL ampicillin sodium salt (Roth, Germany) and 40 µl/plate of 40 mg/mL X-Gal (5-bromo,4-chloro,3-indyl-β-galactopyranoside, Peqlab, Germany solved in 40 mg/mL dimethylformamide, Roth, Germany). Ampicillin selects for bacteria that harbour the Topo plasmid. The X-Gal differentiated between colonies that had the Topo plasmid with an insert (white and light blue) and those that had the Topo plasmid without an insert (dark blue). After a 24 h incubation at 37°C, only white or light blue colonies were picked and inoculated into 5 mL of LB broth (10 g/L tryptone (Becton Dickenson and Company, USA), 50 mg/mL ampicillin sodiumsalt (Roth, Germany), 5 g/L yeast extract, 5 g/L NaCl). These cultures were incubated over night. An aliquot of this cell suspension was directly used as template (3 µl) in a PCR-reaction with the specific primers to check the success of the transformation. The PCR program was only slightly altered (10 min at 94°C in the beginning) to break down the cells and to release the plasmid. The amplified fragments were again checked by agarose gel electrophoresis. Plasmids were isolated from an aliquot of the suspension (Wizard Plus SV Minipreps DNA Purification System, Promega, USA) and sent to sequencing (MWG, Germany) to verify the correct sequence of the insert. Aliquots of the bacteria suspension were frozen in 40 % glycerol and stored at -80°C until the probe preparation for the first spotting event. Then, aliquots were thawed and again inoculated into 5 mL of LB broth (10 g/L tryptone (Becton Dickenson and Company, USA), 5 g/L yeast extract, 5 g/L NaCl, 50 mg/mL ampicillin sodiumsalt, Roth, Germany). These cultures were incubated over night. Plamid preparations were done with the Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA). The plasmid was used as template for the PCR amplifying the insert by means of specific primers and subsequently purified (QIAquick PCR Purification Kit, Qiagen, Germany). The concentration was determined spectrophotometrically (λ = 260 nm). Afterwards the probes were lyophilized for easily adjusting the concentration to 800 ng/µL H_2O. These probes were 1 time diluted in 2 x spot buffer (6 x SSC, 3 M Betain, provided vom S. Thieme, Technical University Dresden) to obtain an amount of 400 ng DNA per 1 µL of 1 x spot buffer.

2.9.3 Slide coating

The first set of slides, Superfrost® Plus (Menzel, Germany), was purchased from Roth (Germany) and coated with poly-L-lysine (Sigma, Germany) according to the protocol from the University of Stanford available in the Internet (http://cmgm.stanford.edu/pbrown/protocols/1_slides.html). In brief, slides were pre-cleaned for 2 h on an orbital shaker at 50 rpm (IKA, Germany) in a cleaning solution containing 60 % EtOH (MERCK, Germany) and 2.5 M NaOH. Then slides were rinsed 5 times in autoclaved, filtered, purified deionized water. Afterwards slides were incubated for 30 min on an orbital shaker in coating solution containing 11.1 % [v/v] poly-L-lysine and 11.1 % PBS (w/o Ca^{2+}, Mg^{2+} from Biochrom, Germany) in autoclaved, filtered, purified deionized water. After rinsing in autoclaved, filtered, purified

deionized water, slides were centrifuged, dried at 45°C, and stored in a dry and dark place. Slides were used within 2 to 3 month. The second set of slides (Gold Seal, Fisher Scientific, USA) were coated following the same procedure. Slides were used within 4 to 5 month.

2.9.4 Spotting

The probes (obtained as described in 2.9.2) were spotted onto the slides prepared as described in 2.9.3 with the Microgrid II spotter (Genescan, USA) at the University of Technology in Dresden. Spotting onto the first set of coated slides was done with the use of four pins. Four replicates of each probe were spotted onto the slide surface. Additionally, a second array (duplicate) was spotted on each slide. Slides were dried over night in the spotter. Afterwards the slides were baked at 80°C for 2 h to fix the phosphate backbone of the DNA to the amino groups of the poly-L-lysine. This was followed by two washing steps in 0.1 % SDS and the denaturing of the DNA by incubating in boiled water for 3 min. The water was removed by dipping in -80°C cold ethanol (absolute, purchased from AppliChem, Germany) and then spin-dried for 5 min at 200 x g. A subsequent blocking of the slide surface with a mixture of 6 % sodium borate solution (1 M Boric acid adjusted to pH 8.0 with NaOH) and 94 % [v/v] 1-methyl-2-pyrrolidon/succinic acid anhydride solution (4.8 M 1-methyl-2-pyrollidon, 0.18 M succinic acid anhydride) was performed. After 30 min incubation, the slides were rinsed in boiled water and then dipped in cold ethanol and spin-dried for 5 min at 200 x g. The spotted slides were stored in a dark and dry place until hybridization.

The second spotting onto the second set of coated slides was carried out with a 12 pin head. Three replicate spots of each probe were spotted a second time on the bottom part of the same slide (six replicates of every gene in total). The post-treatment of the spotted slides differed from the first set in that a subsequent blocking of the slide surface was not performed.

2.9.5 Quality control with SYBRgreen II

A simple and time-effective procedure to determine the quality of the spots was performed using SYBRgreen II as described in Battaglia *et al.* (2000). The fluorophor SYBRgreen II has a specific affinity for single stranded DNA (ssDNA). Molecular probes, where the SYBRgreen II was purchased from, provides a protocol for staining microarrays with this nucleic acid stain. Briefly, SYBRgreen II stain was diluted 10,000 times in 1xTBE (90 mM Tris, 90 mM Boric acid, 2 mM Na_2EDTA, (pH 8.0) and 60 µL of this solution were hybridized onto the array for ca. 2 – 3 min by placing a drop beside the spots and evenly distributed by covering with a cover slip. Afterwards the slide was washed 3 times for about 2 min in 1xTBE buffer and spin-dried for 5 min at 200 x g. The fluorescence was examined with an excitation wavelength of 531 nm and an emission wavelength of 554 nm. Although SYBRgreen II has a fluorescence excitation maximum at λ=494 nm and a fluorescence emission maximum at λ=521 nm. The 513/554 pair

was the one available and was found to work well. To remove the fluorophor from the array, the slides were incubated at room temperature for one hour in a solution containing 1 % [w/v] SDS, 10 mM Tris, 1 mM EDTA (pH 7.5). After drying the slides, the removal of the SYBRgreen II was checked by scanning again. Afterwards the slides were used in hybridization experiments. In the first set of spotted slides only one slide was exemplarily tested in order to check the procedure. In the second set of spotted slides, every slide was checked for quality in this way.

2.9.6 Sample preparation for hybridization

Total RNA was isolated from 24 x 10^6 hepatocytes for each sample with either RNeasy Midikit according to the manual from Qiagen (Germany) or with TRIZOL® reagent (Invitrogen, USA) (Method described in Chomczynski & Sacchi, 1987). The RNA concentration was determined spectrophotometrically (λ = 260 nm). With TRIZOL®isolated total RNA, a DNA digest with RNAse-free DNase (Roche, Germany) was performed for purification. To 18 µL volume containing up to 100 µg total RNA were added 20 U DNase and 4 µL 10 x D-buffer (200 mM Tris-HCL, 20 mM $MgCl_2$, 500 mM KCl_2, sterile filtered). This mixture was incubated at 25°C for 15 min. The success of this procedure was checked by means of gel electrophoresis as described in Chapter 2.7.1. Total RNA was precipitated with 0.5 vol 7.5 M ammonium acetate (Sigma, Germany) and 2.5 vol absolute ethanol at -20°C over night, then centrifuged (30 min at 4°C, 13600 x g), washed with 80 % (v/v) ethanol, dried and re-suspended in RNase free water. A final concentration of 30 µg/7 µL total RNA in water was adjusted.

2.9.7 Labelling

The direct labelling method was used (according to the protocol provided from the FlyChip project http://www.flychip.org.uk/Project/Protocols/crna_processing/direct_labelling.php with minor changes). Thus, during the reverse transcription the Cyanine 3 or 5 dye-labelled deoxynucleotid dCTP was incorporated. The reverse transcriptase SuperScript II (200 U/µL, Invitrogen, USA) and the buffer provided with the enzyme were used as well as Ribonuclease Inhibitor (40 U/µL) from Fermentas (Germany) and customized oligo dT primer (T 18, synthesized from Invitrogen, Germany). First strand cDNA synthesis was performed in amber tubes (Brand, Germany) with a final volume of 30 µL using the following final concentrations: 1 µM oligo dT primer, 25 µg total RNA, 1.3 U Ribonuclease Inhibitor, 13.3 U SuperScript II, 0.1 mM Cyanine 3/5-dCTP (Perkin Elmer, USA), 0.5 mM of each dATP, dGTP, dTTP and 0.2 mM dCTP (dNTP Set from Fermentas, Germany). The reaction was carried out at 42°C for 2 h in the dark. Subsequently, the remaining RNA was hydrolyzed by adding 10 µL of 0.5 mM EDTA and 10 µL of 1M NaOH and incubation at 65°C for 15 min. Afterwards the samples were cooled to room temperature and then neutralized with 25 µL of 1 M Tris-HCL (pH 7.5). The labelled samples were immediately processed or stored at -20°C until use.

Sample clean-up was performed using Microcon YM-30 columns (Millipore, USA) in order to remove residual components of the synthesis reaction. The reaction mixture was placed in a Microcon column and diluted in 450 µL TE buffer (10 mM Tris, 1 mM Na_2EDTA). After centrifugation (10 min, 13600 x g), this washing step was repeated and the probes again centrifuged for 12 min at 13600 x g. The column was inverted and the content was caught in a new tube. The remaining solution containing only the labelled cDNA fragments was brought up to 70 µL with TE buffer and placed in a micro UV-cuvette (Brand, Germany). The DNA content (260 nm) and Cyanine 3 (550 nm) or Cyanine 5 amount (650 nm), respectively, were measured photometrically. The calculation of cDNA amount and cyanine dye incorporation efficiency during reverse transcription was done with the aid of the equation provided on the website http://www.pangloss.com/seidel/Protocols/percent_inc.html. The equation uses Beer's Law and the extinction coefficient of the Cyanine dyes to calculate the amount of Cyanine dye in a sample. The concentration is then used to calculate the mass of the synthesized cDNA and of Cyanine-labelled nucleotides, respectively. The ratio of the mass of Cyanine-labelled nucleotides incorporated into the mass of cDNA synthesized is called the incorporation efficiency. Additionally, the base/dye ratio (equation 1) and the pmol of incorporated dye (equations 2 and 3) were calculated according to the following equations (refer also to Table 2.2; calculated values can be found in the appendix).

$$\text{base/dye ratio} = \frac{(A_{260} - A_{dye} \cdot \text{dye correction factor}) \cdot \text{extinction coefficient}_{dye}}{A_{dye} \cdot \text{extinction coefficient}_{base}} \quad (1)$$

$$\text{Cy3 pmol} = \frac{A_{cy3} \cdot \text{total volume of probe}}{0.15} \quad (2)$$

$$\text{Cy5 pmol} = \frac{A_{cy5} \cdot \text{total volume of probe}}{0.25} \quad (3)$$

Table 2.2 Required data for incorporation calculations[1]

	Absorbance maximum [nm]	Extinction coefficient	Dye correction factor
Cy 3	550	150000	0.08
Cy 5	650	250000	0.05
Base	260	8919	-

[1] according to the homepage from Ambion http://www.ambion.com/techlib/append/base_dye_details.html

A base/dye ratio of 12-25 is recommended by Molecular Probes as well as at least 15 pmol Cyanine dye per slide by several protocols available in the internet (e.g. http://www.bri.nrc.gc.ca/pdf/microarray_Direct_Incorporation_Labeling%20_Protocol_e.doc).

The corresponding samples were combined and 1 µL yeast tRNA (20 µg/µL, Invitrogen, USA) was added to each probe, because it blocks non-specific DNA hybridizations. Afterwards the combined hybridization samples were dried in a speed vac (system containing Univapo 100 Ech, Unicryo MC2L, Univac from Uniequip, Germany).

Each sample RNA and the corresponding control RNA from one experiment were labelled once with Cy3 and once with Cy5, respectively. Both Cy3-sample RNA and Cy5-control RNA as well as Cy5-sample RNA and Cy3-control RNA were combined so that a switch of the labelled dyes was performed. Two array slides were used for this dye swap and treated as a replicate.

Furthermore, a hybridization control was produced. The fragments of a Lambda DNA *Hin*dIII Digest (commercially available from Sigma, Germany) were used as template in a linear PCR to amplify only one strand of the 564 bp fragment using the reagents from Taq Core PCR Kit (Qiagen, Germany), the primer with the sequence 5' AGAGCGATTTATTCTTCTGAA 3' and Cyanine 3/5 labelled dCTP. An aliquot of this labelled fragment was added to the combination of the labelled samples and the yeast tRNA before it was dried in a speed vac.

2.9.8 Hybridization

The dried samples were re-suspended in 35 µL hyb-buffer (composition is provided below), kept at 92°C for 2 min and then centrifuged (1 min at 13600 x g). Samples were placed beside the spotted area. With a cover slip (Hybri-Slips, Sigma, Germany) the probe was evenly distributed over the spots. Then the slide was placed in a hybridization chamber (X-Hyb, HTI bio-X GmbH, Germany) with a humidified atmosphere and incubated at 42°C or 65°C, respectively for at least 16 h in a water bath (grant Instruments, Germany). The hybridization temperature was depending on the applied hybridization buffer (see below).

Initial experiments were done with the commercial ArrayHyb™ Microarray hybridization buffer (Sigma, Germany). The temperature of hybridization with this buffer was 65°C. After

hybridization, the slides were cleaned from the Hyb-buffer with three washing buffers according to the manual: wash buffer 1 (1 x SSC, 0.03 % [w/v] SDS), wash buffer 2 (0.2 x SSC), wash buffer 3 (0.05 x SSC).

One problem occurring with the commercial ArrayHyb™ Microarray hybridization buffer was that the cover slips were very difficult to remove after hybridization. Thus, subsequent hybridizations were carried out with a self-prepared hybridization buffer (5 x SSC, 0.1 % [w/v] SDS, according to Bartosievicz *et al.* (2001) without formamide). The slides were cleaned from Hyb-buffer with the following buffers: wash buffer 1 (preheated at 42°C) (2 x SSC, 0.1 % [w/v] SDS), wash buffer 2 (0.2 % [w/v] SDS), and wash buffer 3 (0.1 x SSC). Each washing step was performed for 6 min during which the slides were shaken gently. After the wash steps the slides were centrifuged at 200 x g for 5 min and subsequently immediately scanned, or stored in the dark until scanning.

2.9.9 Scanning

The hybridized slides were scanned at a 10 µm resolution with the ScanArray Express Scanner (Microarray Scanner Packard Bioscience, Perkin Elmer, USA). The laser power and PMT values were adapted manually to each slide with the intention to reduce the background and to avoid saturated signals.

2.9.10 Data analysis

The software ScanArray Express (Perkin Elmer, USA) provides the measured fluorescent intensities as raw data and the same set of data LOWESS normalized. The data were exported into Microsoft Excel and there processed manually as follows: At first, the quality criteria signal/noise ratio was applied. This ratio has to be ≥ 2. That means, the signal of the spot has at least a 100 % greater intensity than the background around the spot. From this it follows that all spots with a signal/noise ratio < 2 were not taken into account for further analysis. From here, two methods of normalization were used. Normalization is necessary because of several factors (e.g. the different incorporation efficiencies and quantum yields of the two used fluorescent dyes) have to be taken into account to distinguish impacts due to the methods from the biologically relevant effects. The first method needed a deduction of the background intensity from the spot intensity and with this value, the normalization via house-keeping genes was carried out. As house-keeping genes β-actin, cytochrome-c-oxidase subunit VIa and the poly $(dA)_{18}$ oligonucleotide were used. From each spot of these three fragments the ratio of Cy5/Cy3 was calculated and averaged. With this factor, the Cy3 intensities of all other spots were normalized. Afterwards, the fluorescent intensities of the replicate spots of all genes of

interest were averaged and a value for gene expression was calculated with the following equations (4, 5). A gene was considered to be down-regulated, if the equation 4 was fulfilled, otherwise it was considered to be up-regulated, if the equation 5 is fulfilled.

$$-1 < \log_2 \frac{Cy3_{normalized}}{Cy5} \tag{4}$$

$$1 < \log_2 \frac{Cy3_{normalized}}{Cy5} \tag{5}$$

The second method used the mathematical algorithm LOWESS (Cleveland, 1979) which presumes that the majority of the spots are not regulated, i.e. most spots have the same fluorescent intensity of the Cy dyes. Due to this assumption and the amount of the spots, a locally weighted regression and a smooth curve fitting are performed. These normalized data were then further analyzed using the equations 4 and 5.

Chapter 3: Results and Discussion

Chapter 3.1

Application of a cell viability assay prior to gene expression analysis in primary hepatocytes from rainbow trout

Introduction

Primary fish hepatocyte cultures are indispensable tools for studying metabolic activity (Moon *et al*, 1985; Segner & Cravedi, 2001) as well as mechanisms of toxicity *in vitro* (Klaunig, 1984; Pesonen & Andersson, 1997). An important incentive to the use of fish hepatocytes as model systems is the role of the liver as major site of metabolism and as a hormone target site. One significant advantage of hepatocyte cultures over the use of whole fish is that the *in vitro* cultures allow the role of selected parameters to be investigated in a well-defined, controlled environment. As well, a single fish can yield millions of cells allowing numerous parameters to be investigated without having to sacrifice large numbers of animals. Among the donor species used most prominently for hepatocyte isolation is rainbow trout.

Primary hepatocyte cultures from rainbow trout are widely applied for detecting and understanding adverse effects elicited by ecotoxicants. For example, they have frequently been employed to study the induction of cytochrome CYP1A upon exposure to dioxin and related compounds, acting as ligands of the aryl hydrocarbon receptor (Pesonen & Andersson, 1991; Navas & Segner, 2001; Rabergh *et al*, 2000; Behrens *et al*, 2001). As well, rainbow trout hepatocytes have been suggested for studying estrogenic effects because of the *in vitro* expression of an active estrogen receptor (Flouriot *et al*, 1993) and the inducibility of typical down-stream effects such as the synthesis of vitellogenin (Pelissero *et al*, 1993; Sumpter & Jobling, 1995). Initially, potentially harmful effects of ecotoxicants were studied on the level of protein abundance or enzyme activity but more recently are increasingly explored on the level of gene expression (Ren *et al*, 1996; Gagne & Blaise, 1998). Central to all these investigations is the determination of the state of viability of the hepatocyte cells either alone or in the presence of the ecotoxicant(s) under study. This is important because changes to cell viability can affect gene expression and/or protein function but as well, changes in gene expression

and/or protein function can eventually lead to alterations in cell viability. Thus, correlating cell viability to specific gene expression or protein function analysis can add valuable information regarding ecotoxicant effects and is important for interpreting the *in vitro* results.

Several assays have been employed to assess primary hepatocyte viability. Neutral red, which accumulates in lysosomes of viable cells, has been applied in primary cultures of mammalian (Zhang *et al*, 1990) and trout hepatocytes (Risso-de Faverney *et al*, 2001). The use of propidium iodide, which enters cells upon membrane damage and intercalates with the DNA, has been particularly advanced by Gagne & Blaise (1997; 1996). Further, reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide) to the blue formazan by viable cells was employed in isolated trout hepatocytes (Ferraris *et al*, 1998; Ferraris *et al*, 2002; Ernst *et al*, 2001). Finally, leakage of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium has been used as an indirect measure of hepatocyte viability (Ferraris *et al*, 1998; 2002; Pesonen & Andersson, 1992; Rabergh *et al*, 2000). Among these viability assays, the LDH assay is the only one which can be used without the necessity to sacrifice the cells. However, this test evaluates cell viability in an indirect, non-specific way and the procedure is rather cumbersome if applied to multiwell tissue culture plates.

It thus was the goal of this chapter of this thesis to adopt a simple, directly applied, non-invasive cell viability assay to primary cultures of rainbow trout hepatocytes. The method of choice was a combination of two fluorescent indicator dyes. AlamarBlue™ was used as a measure of cellular metabolic activity and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) as an esterase substrate indicative of cell membrane integrity. The two dyes had previously been shown to work well together for observing viability of fish cell lines (Schirmer *et al*, 1997; Dayeh *et al*, 2002b; 2003) and long-term cultures of porcine lenses (Oriowo *et al*, 2002).

Three steps were pursued in order to achieve the goal of establishing the use of alamarBlue and CFDA-AM in the primary rainbow trout hepatocytes. First, the two dyes were used together to repeatedly monitor the viability of hepatocyte cultures over 96 hours in serum free L-15 or M199 culture medium. Second, to examine if the two dyes were capable of detecting an insult to the hepatocytes by a toxicant, pentachlorophenol (PCP)-exposed cultures were monitored using alamarBlue/CFDA-AM. Finally, 17β-estradiol (E_2) treated hepatocytes were investigated for their viability prior to RNA extraction, and gene expression in these cells compared to cells not pre-treated with alamarBlue/CFDA-AM. This was done in order to explore if the fluorescent indicator dyes could be applied to monitor primary rainbow trout hepatocyte viability just prior to analysis of gene expression without by themselves altering mRNA abundance.

Results

A combination of two fluorescent indicator dyes, focussing on cellular energy metabolism and cell membrane integrity, was established as a versatile means of quantifying cell viability in primary rainbow trout hepatocyte cultures. In the following, the usefulness of the cell viability assays is presented according to the different scenarios of application explored in the course of this work.

Cell viability in different culture media

Two media commonly used for culturing primary rainbow trout hepatocytes, M199 and L-15, were studied with regard to their ability to support cell viability over up to five days. Cell viability was judged based on microscopic observations and quantitative evaluation using the fluorescent indicator dyes. Both media were found to support the formation of aggregates within 24 h of plating although the morphology of the aggregates differed somewhat. In M199 medium, cells could no longer be differentiated but remained more distinct in L-15, an observation that was made throughout the remaining hepatocyte culture period (Figure 3.1.1)

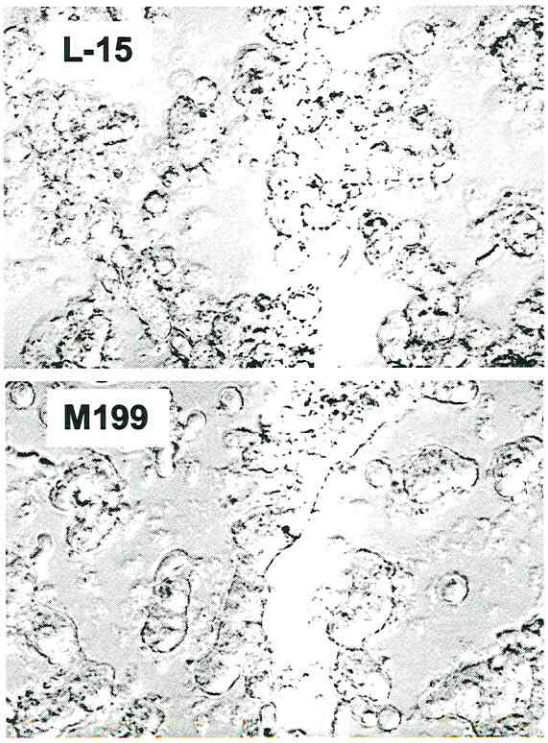

Figure 3.1.1 Typical appearance of hepatocytes in L-15 and M199 culture media (magnification x400). In L-15 medium, the cells appeared round within the aggregates, while in M199 single cells could no be identified in the aggregates.

The 24 h time point was also used as the reference point for quantitative cell viability assessment. In five independent hepatocyte isolations stemming from four female and one male fish, both alamarBlue and CFDA-AM yielded fluorescent unit readings that were 10 to 20-fold above the background values without cells. In general, the fluorescent unit readings for each dye were similar in the two media and variation among replicate culture wells was less than 10 %. Past the 24 h time point, aggregation continued until the termination of culture at 96 h, implying that the cells stayed viable. This was also confirmed by the cell viability assays although some general differences were noticeable between the two media and the two fluorescent indicator dyes (Figure 3.1.2). With regard to the media, the cell viability assays suggested L-15 as a medium that more stably supported hepatocyte viability in independent culture preparations and this medium was therefore used in all subsequent experiments. In terms of the indicator dyes, alamarBlue appeared to more sensitively respond to changes in cellular function as judged by the greater range of fluorescent unit readings observed for this dye in independent hepatocyte preparations cultured in M199.

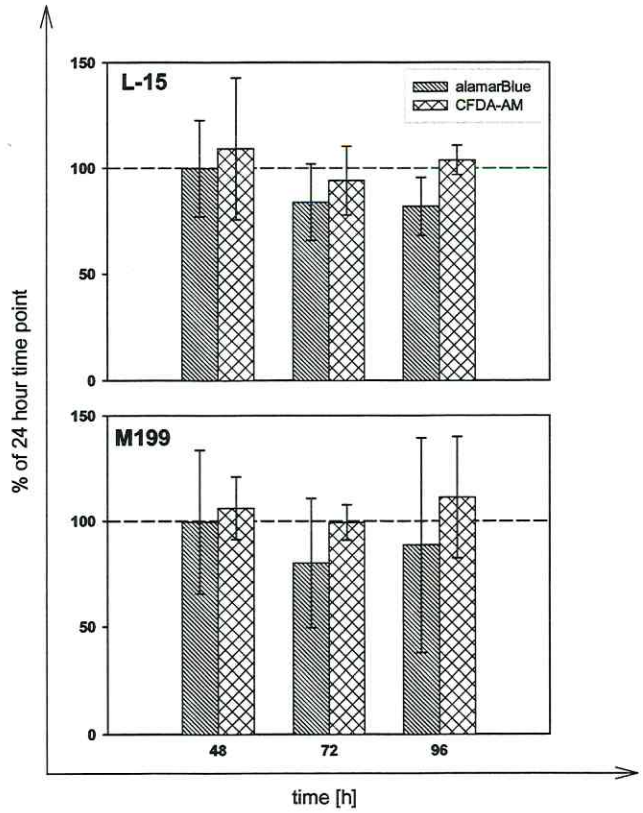

Figure 3.1.2 Cell viability of hepatocytes cultured in L-15 and M199, respectively, over a course of 96 h. Data are given as mean ± standard deviation of triplicate wells in percent relative to the 24 h time point for each of the fluorescent dyes, alamarBlue and CFDA-AM. Reference line represents 100 % cell viability at time point 24 h.

In a separate experiment, hepatocytes were plated in L-15 in Non-Primaria plates coated with Matrigel™. In this culture environment, cells initially attached but dislodged from the surface after 24 h and did not form aggregates. Indeed, whereas the cell viability assays yielded fluorescent units that were about 10-fold above background for both alamarBlue and CFDA-AM at the 24 h time point, fluorescent units for alamarBlue dropped to background levels 24 h later (48 h of culture). For CFDA-AM, the drop in fluorescent units was about 40 % at the 48 h time point. These results confirm the ability of the fluorescent indicator dyes to distinguish intact from impaired hepatocytes and the greater sensitivity of alamarBlue in detecting changes to cellular function.

Cell viability upon exposure to PCP

To explore the ability of alamarBlue and CFDA-AM to identify a decline in cell viability due to a toxic insult, hepatocytes of two fish were incubated for 24 h up to $25x10^{-6}$ M of PCP (Figure 3.1.3). AlamarBlue in both hepatocyte cultures indicated an impairment of cellular metabolic activity with EC_{50} values amounting to about $21x10^{-6}$ M PCP in Figure 3.1.3 A and $13x10^{-6}$ M PCP in Figure 3.1.3 B. In contrast, CFDA-AM only in one case revealed an impact on the cells and EC_{50} values could not be calculated (Figure 3.1.3 A/B). Exposure of subsequent hepatocyte cultures to 10^{-4} M of PCP confirmed the differences in alamarBlue and CFDA-AM readings. In these experiments, fluorescent unit readings were, compared to the control, 7±0.3 % for alamarBlue and 62±17 % for CFDA-AM (average of four individual culture wells of four independent hepatocyte preparations). Finally, when the two fluorescent indicator dyes were applied to PCP-exposed cells of the rainbow trout liver cell line, RTL-W1, instead of the primary hepatocytes, both dyes indicated cellular damage to the same extent. For the cell line, EC_{50} values were $19±3x10^{-6}$ M PCP and $22±5x10^{-6}$ M PCP (average of five independent experiments) for alamarBlue and CFDA-AM, respectively, and thus were in the range of those observed for alamarBlue in the primary rainbow trout hepatocytes.

In addition to the quantification of cell viability using the fluorescent indicator dyes, cells were observed microscopically. In general, PCP led to distinct cell lifting but concentration-dependent morphological differences were not visible by eye.

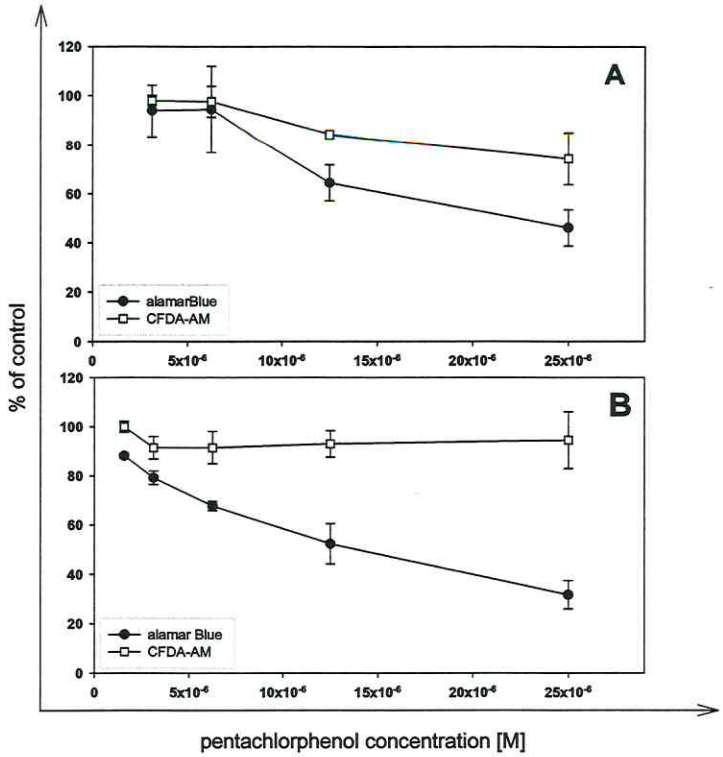

Figure 3.1.3 Effect of pentachlorophenol after 24 h exposure on cell viability in primary hepatocyte cultures. Data are given as mean ± standard deviation of 3 replicate wells (**A**) or 4 replicate wells (**B**) in percent relative to the control (no treatment, which was similar to the DMSO solvent control) for each of the fluorescent dyes, alamarBlue and CFDA-AM.

Cell viability upon exposure to E_2

Exposure of hepatocytes to E_2 for up to 78 h led to no or little change in fluorescent unit readings compared to the control for alamarBlue and CFDA-AM for up to 10^{-5} M E_2 (Figure 3.1.4). A decline in the % of control was observed only when 10^{-4} and 10^{-3} M E_2 were employed. At these concentrations, fluorescent unit readings were occasionally found to drop up to 50 % for alamarBlue and 30 % for CFDA-AM.

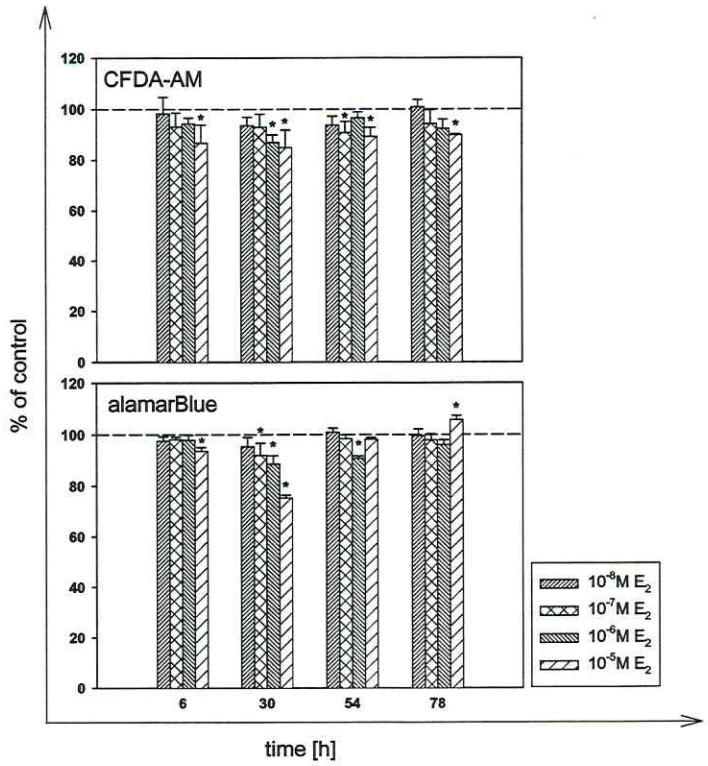

Figure 3.1.4 Time course of the effects of E_2 on hepatocytes from a female immature rainbow trout cultured in L-15 on cell viability. Reference line represents 100 % cell viability measured in solvent control (0.5 % EtOH v/v). Data are given as mean ± standard deviation of 3 replicate wells in percent relative to the solvent control. Asterisks indicate differences compared to ethanol (ANOVA followed by Dunnett test, $P<0.05$)

The viability of cells upon exposure up to 10^{-6} M E_2 was confirmed by the secretion of VTG protein into the culture medium as an indicator of hepatocyte differentiated function. Maximal levels of induction (up to approximately 20-fold) were found between 30 to 78 h of exposure and at 10^{-7} M E_2 for female and 10^{-8} to 10^{-7} M E_2 for male rainbow trout.

Potential impact of cell viability assays on gene expression (mRNA abundance)

Because of its importance as a marker of hepatocyte differentiation and its role in endocrine function, VTG was subsequently used as a specific target gene for exploring the suitability of carrying out the alamarBlue/CFDA-AM cell viability test just prior to the analysis of gene expression using quantitative real-time rt-PCR. Investigations were further supported by a non-targeted, random gene expression analysis approach, differential display rt-PCR. For these studies, parallel sets of cells were thus treated as desired before they either were directly processed for PCR analysis or underwent a prior 30 min alamarBlue/CFDA-AM exposure.

Targeted gene expression analysis – Hepatocytes exposed from 10^{-8} to 10^{-5} M E_2 for 78 h and then either treated with alamarBlue/CFDA-AM or not yielded comparable levels of VTG mRNA abundance. One example of this is shown in Figure 3.1.5. Differences generally were less than 30 % and no pattern of under- or overestimation for alamarBlue/CFDA-AM treated- or untreated cells was observed. For comparison, the standard error of the mean of normalized expression data of three replicate wells with identical cDNA probes mostly varied between 0 and about 12 % (resembled by error bars in Figure 3.1.5). Considering the higher variability of independent PCR analyses compared to the variability of independent cDNA syntheses of identical cell cultures (Ackermann, 2000), it can be assumed that the 30 % variability are caused by the method rather than by specific effects of the dyes on VTG (and β-actin) mRNA abundance.

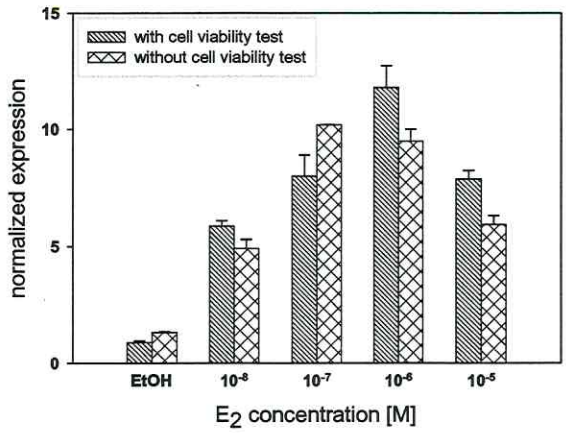

Figure 3.1.5 Comparison of VTG mRNA abundance upon exposure of primary hepatocytes from a female rainbow trout for 78 h to various levels of E_2 with or without a 30 min alamarBlue/CFDA-AM exposure. Detectable VTG mRNA level in the control indicates a beginning vitellogenesis in this female. Each bar represents the mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2.

Random gene expression analysis – Differential display rt-PCR using different combinations of fluorescently labelled primers and several exposure conditions revealed no apparent differences in the expression patterns of cells that were or were not exposed to alamarBlue/CFDA-AM for 30 min prior to RNA extraction (Figure 3.1.6). Thus, the random differential display analysis approach confirmed that alamarBlue/CDFA-AM did not impact on the pattern of gene expression, as had been observed for the target gene analysis.

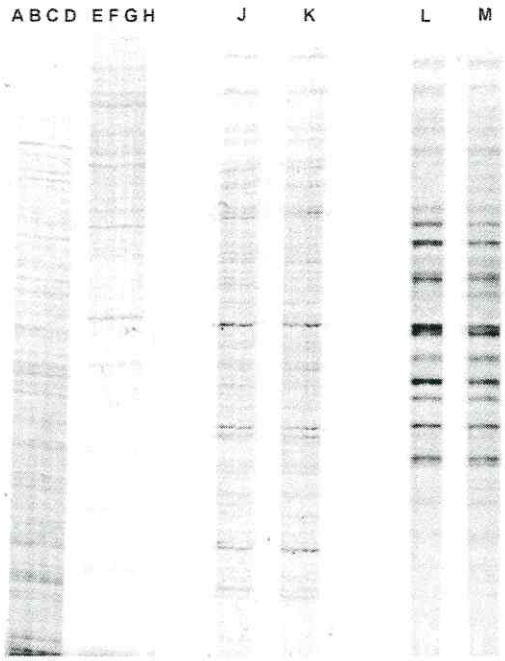

Figure 3.1.6 Comparison of gene expression patterns in RTL-W1 cells (lanes A-H) and primary rainbow trout hepatocytes from a female fish (lanes J-M) with or without a 30 min alamar Blue/CFDA-AM exposure just prior to processing for differential rt-PCR. PCR products were separated on a polyacrylamide gel and visualized using the BioRad Molecular imager. Only portions of the entire gel were selected for each respective comparison. PCR was carried out with fluorescently labelled primers as follows: Lanes A-D (blank cells) - primers A2 & A4 with A-B representing RTL-W1 cells without and C-D with alamarBlue/CFDA-AM exposure; Lanes E-H (blank cells), – primer A3 with E-F representing RTL-W1 cells without and G-H with alamar Blue/CFDA-AM exposure; Lanes J-K (EtOH exposed 0.5 %) – primer A3 with J representing primary hepatocytes with and K without alamarBlue/CFDA-AM exposure; Lanes L-M (10^{-6} M E_2) – primer A3 with L representing primary hepatocytes with and M without alamarBlue/CFDA-AM exposure.

Discussion

A combined alamarBlue/CFDA-AM assay was adopted as a simply means to non-invasively monitor cell viability of primary hepatocytes from rainbow trout with or without subsequent analysis of gene expression. AlamarBlue is a commercial preparation of the dye resazurin (O'Brien *et al*, 2000). Upon diffusion into living cells, it is reduced to the water soluble, fluorescent product resorufin by reductases localised in mitochondrial membranes as well as in the cytosol (Gonzalez & Tarloff, 2001). It is increasingly being used in pharmacology for repeated measurement and high throughput screening with mammalian cell lines (Evans *et al*, 2001; McMillian *et al*, 2002). As well, it has recently been validated for primary rat hepatocytes (Slaughter *et al*, 1999; McMillian *et al*, 2002) but not yet for primary hepatocytes of fish. Along the lines of alamarBlue, CFDA-AM diffuses into cells rapidly but is converted by non-specific esterases of living cells from a nonpolar, nonfluorescent dye into a polar, fluorescent dye, 5-carboxyfluorescein (CF), which diffuses out of cells slowly. Esterase substrates have been used as a measure of cell membrane integrity since the 1960s (Rotman & Papermaster, 1966) but CFDA-AM is an example of one development to improve this application (Haugland, 2004). In the study presented here, alamarBlue and CFDA-AM were applied for the first time in primary fish hepatocytes. Both dyes elicited by themselves no impact on the cells and thus allowed for repeated analysis of the same set of cells over the course of an experiment.

Based on the two fluorescent indicator dyes, L-15 cell culture medium was superior in comparison to M199 in supporting *in vitro* cell viability of the rainbow trout hepatocytes. This is reminiscent of Mommsen *et al.* (1994) who noted most consistent results in fish hepatocyte cultures by using L-15. Both M199 (e.g. Pesonen & Andersson, 1991; Pawlowski *et al*, 2000) as well as L-15 (e.g. Klaunig *et al*, 1985; Kocal *et al*, 1988; Ferraris *et al*, 2002) have frequently been used in primary hepatocyte cultures from rainbow trout. M199 contains more vitamins and a higher number of amino acids but in lower concentration than L-15. The latter is commonly used for maintaining fish cell lines (e.g. Lee *et al*, 1993). Aside from the greater consistency of cell viability in L-15, slight differences in the morphology, but not the speed of formation, of monolayers of rainbow trout hepatocyte in L-15 and M199 media were seen. Recently, Kim & Takemura (2003) reported a more rapid monolayer formation in the beginning of culture of tilapia hepatocytes in M199 and William's medium E compared to L-15. However, vitellogenin synthesis as an indicator of hepatocyte differentiated function was highest with L-15 in the tilapia cells. This again is in support of the better sustainability of fish hepatocyte cultures in L-15.

AlamarBlue and CFDA-AM were capable of distinguishing the presence or absence of an insult on rainbow trout hepatocyte viability upon exposure to potentially toxic compounds. For example, 17β- estradiol resulted in a detectable decline of cell viability for both alamarBlue and CFDA-AM only at concentrations of more than 10^{-5} M. As well, a decline in fluorescent unit readings compared to the control was observed for both dyes upon exposure to PCP. However,

the alamarBlue assay reacted much more sensitively to PCP than CFDA-AM. This is in accordance with Dayeh *et al.* (2002a) who found a much steeper decline in alamarBlue fluorescent unit readings compared to CFDA-AM in the rainbow trout gill cell line, RTgill-W1, after exposure for 24 h in L-15/ex to PCP. The application of the two dyes together has the potential of revealing the mechanism(s) behind impaired cell viability as has been variously demonstrated in the past (Dayeh *et al*, 2003; Oriowo *et al*, 2002; Schirmer *et al*, 1998a; 2000). Thus, PCP could be interpreted to particularly impact on hepatocyte metabolic function. Yet, in the example presented here, mechanism-based differences should be cautiously discussed. This is because the rainbow trout liver cell line, RTL-W1, lacked a differing response upon PCP exposure which points, at least in part, to a methodological shortcoming of the assay applied to hepatocytes. In order to limit handling stress in hepatocytes, only half the medium had been removed and replaced in contrast to the cell lines where the complete medium was exchanged without any difficulty. It is conceivable that esterases leaking out of damaged cells or cell fragments containing esterases were still partly present in the portion of the medium not exchanged prior to dye application in the hepatocytes. Thus, these enzymes may have transformed CFDA-AM to its fluorescent form, CF, thereby leading to an over-prediction of hepatocyte cell viability. Along these lines, increased CF fluorescent unit readings were found in preparations of the ciliate *Tetrahymena thermophila* in which leaked out esterases or cell fragments were not removed (Dayeh *et al*, 2004). Future investigations should explore if a more complete medium removal prior to fluorescent indicator dye addition is feasible for the hepatocytes and if this methodological change would remedy the differences in sensitivity observed for PCP.

Application of the two fluorescent indicator dyes did not impact on gene expression patterns in the hepatocytes. This was confirmed by monitoring VTG/β-actin mRNA abundance upon E_2 exposure in primary hepatocytes specifically and by observing differential gene expression patterns in RTL-W1 and primary hepatocytes in control or E_2 treated cells using random primers generally. The two dyes were applied to the cells for 30 min, a time frame sufficient for significant mRNA synthesis should transcription be initiated. Thus, the results presented here indicate that the two indicator dyes do not directly regulate gene transcription and thus are valuable for assessing cell viability just prior to gene expression due to a toxic insult in the primary rainbow trout hepatocytes. Nevertheless, despite the use of the random gene expression monitoring approach, in addition to analysing specific target genes, not all possible gene regulations can be accounted for with the methods applied. In the future, whole genome array analysis, comparing cells with or without prior alamarBlue/CFDA-AM application, would be the method of choice to fully confirm the lack of impact on gene expression by the fluorescent indicator dyes as demonstrated here.

Taken together, a non-invasive cell viability assay consisting of a combined application of two fluorescent dyes was adapted to primary hepatocyte cultures from rainbow trout and showed L-15 culture medium to more stably support cell viability than M199. The fluorescent cell viability assay did not show an impact on gene expression as demonstrated by investigating mRNA abundance of VTG/β-actin by means of qPCR as well as a random set of genes by means of differential display rt-PCR. Thus, the assay should be a useful alternative to the conventional rainbow trout hepatocyte viability monitoring methods. The assay works directly in cells but nevertheless is non-invasive and has the potential to also differentiate more specific mechanisms of toxicity, such as an impact on mitochondria. This is particularly useful when an ecotoxicant or an ecotoxicant mixture is to be assessed with as of yet unknown toxic potency.

Consequences for the following Chapters

In this chapter, L-15 culture medium was found to stably support hepatocyte viability and was thus used for all future investigations related to this thesis. The application of the non-invasive cell viability assay prior to gene expression analysis enables the first assessment of an ecotoxicant with regard to its potential adverse effects on living cells. Thus, this assay was subsequently used as an easy and fast pre-screening tool to detect acutely toxic concentrations as well as specific effects, such as esterase inhibition and potential proliferation of mitochondria, of the synthetic estrogen 17α-ethinylestradiol alone or in combination with the estrogen receptor blocker ICI 182,780 (Chapter 3.2) as well as of the active ingredient of a lipid lowering drug, clofibric acid (Chapter 3.3).

Chapter 3.2

Regulation of estrogen-responsive genes in primary rainbow trout hepatocytes

Introduction

Ecotoxicants can elicit their effects either directly by altering the expression of toxicant-responsive genes or indirectly by requiring a cell to respond to interferences with cellular homeostasis or protection. Thus, deciphering how ecotoxicants act on the molecular and cellular level is a key to deriving "early warning prognostic biomarkers" and to predicting organism response based on causality (Moore, 2002). It is for this reason that advances in ecotoxicology increasingly rely on a mechanistic understanding of ecotoxicant action (Eggen *et al*, 2004). One approach to achieving this is the combination of well defined *in vitro* models, such as primary fish hepatocytes, and sensitive cellular and molecular endpoints, such as the fluorescent-based assessment of cell viability and mRNA expression (Chapter 3.1).

Ecotoxicants can be defined as substances discharged into the environment through human actions and having the potential to impact on ecosystems at relatively low concentrations (Connell *et al*, 1999). One such group of chemicals are estrogen-mimicking compounds, such as the contraceptive 17α-ethinylestradiol (EE_2) which has been detected in the aquatic environment in substantial amounts (Kümmerer, 2001). Like the natural hormone 17β-estradiol (E_2), which is also significantly discharged into the environment, EE_2 is designed to bind to and activate the human estrogen receptor. Due to the conserved nature of steroid hormone receptors, however, E_2, the synthetically-derived EE_2, as well as other estrogen mimics, also activate the estrogen receptor in other vertebrates, such as fish (Le Drean *et al*, 1995; Nimrod & Benson, 1997; MacKay *et al*, 1996). The induction of the egg yolk protein, vitellogenin (VTG), is one of the best studied early responses initiated upon estrogen receptor activation in the liver of oviparous fish and has been suggested as an *in vivo* and *in vitro* biomarker for exposure of fish to estrogen-mimicking compounds (Jobling & Sumpter, 1993; Pelissero *et al*, 1993; Sumpter & Jobling, 1995). More recently, the vitelline envelope proteins (VEPs) have been discussed as more sensitive indicators of exposure to estrogens and potential harmful ecotoxicant effects (Arukwe *et al*, 2000; Celius & Walther, 1998).

Aside from VTG and VEPs, however, other genes or their protein products have been identified as being regulated upon exposure to estrogen or estrogen-mimicking compounds to fish. For

example, the cytochrome CYP3A27 was found to be down-regulated both on the transcriptional as well as the translational level in the liver of male and female rainbow trout injected intraperitoneally (i.p.) with E_2 (Buhler *et al*, 2000). Furthermore, the expression of the gene coding for the serum protein transferrin was found to be down-regulated upon i.p. injection or water-based exposures of sheepshead minnow to E_2 (Denslow *et al*, 2001; Larkin *et al*, 2003a; 2002). However, never has this variety of genes been investigated in one model. Extrapolating between model systems is difficult due to the different levels of complexity. Additionally, the model systems vary with respect to their availability generally and to their suitability for follow-up mechanistic investigations specifically. For example, although inhibitor-based studies are possible *in vivo*, they can be controlled much more effectively by *in vitro* cellular approaches.

In light of these considerations, the regulation of selected estrogen-responsive genes upon E_2 and EE_2 exposure is explored in a single model, primary hepatocytes of rainbow trout. Target genes, which were selected based on previous reports using different species and exposure scenarios, were studied for their time- and concentration-dependent expression, and the dependence of gene regulation on the estrogen receptor was explored using the receptor antagonist ICI 182,780 (Wakeling & Bowler, 1992). Gene regulation was monitored based on mRNA abundance using quantitative real-time reverse transcription polymerase chain reaction (qPCR). Previously, the detection of mRNA was shown to reliably indicate the effects of estrogenic substances upon short-term *in vivo* or *in vitro* studies (Scholz *et al*, 2004), and qPCR was shown to be of advantage due to its sensitivity (Celius *et al*, 2000).

Results

The goal of this study was to explore the expression of eight genes upon exposure to 17β-estradiol (E_2) and 17α-ethinylestradiol (EE_2) in one single model, primary rainbow trout hepatocytes. The eight genes were β-actin, VTG, VEPs α, β, γ, serum albmin, transferrin and CYP3A27. In the following, the expression of each of them is illustrated first with regard to basal levels, followed by changes to mRNA abundance due to E_2 or EE_2 exposure. In order to identify the role of the estrogen receptor in all these responses, the estrogen receptor antagonist, ICI 182,780, was applied. To ensure that gene expression was not impacted upon by impaired cell viability, potential adverse effects were first explored by means of a combination of two fluorescent indicator dyes.

Cell Viability

Primary hepatocyte cultures were monitored for cell viability over the course of the experiment (up to 96 h) at each time point immediately prior to RNA isolation, using the fluorescent indicator dyes alamarBlue, for metabolic activity, and CFDA-AM, for general membrane integrity (Chapter 3.1). Consistent fluorescent unit values in cells without any treatment (blank) as well

as in the ethanol control indicated no alteration in cell viability. Furthermore, fluorescent unit readings showed little variation with increasing E_2 or EE_2 concentrations (see also Chapter 3.1). In contrast, the inhibitor ICI 182,780 at 10^{-5} M, alone or in combination with EE_2 (10^{-7} M) or E_2 (10^{-6} M), yielded a decline in cell viability of about 25 to 40% in the CFDA-AM assay (Figure 3.2.1). As a consequence, ICI 182,780 concentrations above 10^{-6} M were not considered in gene expression studies with the inhibitor. From the cell viability studies it was concluded that gene expression analysis could be performed on these cells without having to consider alterations due to acute toxicity.

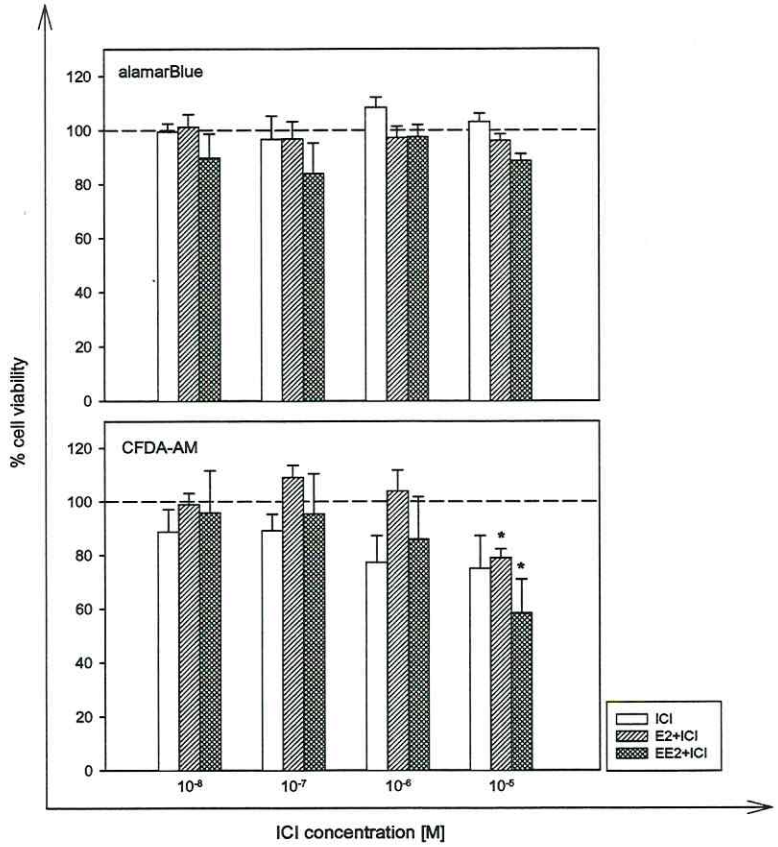

Figure 3.2.1 Effect of ICI 182,780 alone or in combination with EE_2 or E_2 on cell viability after 78 h of exposure. Data are given as mean ± standard deviation of triplicate wells in percent relative to the solvent control for each of the fluorescent dyes, alamarBlue and CFDA-AM. Reference line represents the solvent control for each respective exposure scenario. One of two independent experiments is shown. Asterisks indicate a significant decrease compared to the solvent control (ANOVA followed by Dunnett test, $P<0.05$).

β-actin

The house-keeping gene β-actin showed no significant alterations in mRNA expression levels. Summarizing all data, neither the treatment with E_2 (36 independent PCR well plates) (Figure 3.2.2 A) nor EE_2 (14 independent PCR well plates) (Figure 3.2.2 B) in varying concentrations had a significant influence on the mRNA amount of β-actin. As well, β-actin was not affected by the duration of culturing (Figure 3.2.2 A). Because of the lack of alterations due to the treatments, the range in threshold cycle values (from 18 to 30) is attributable to the deviation between either individual fish or independent cDNA syntheses and PCRs.

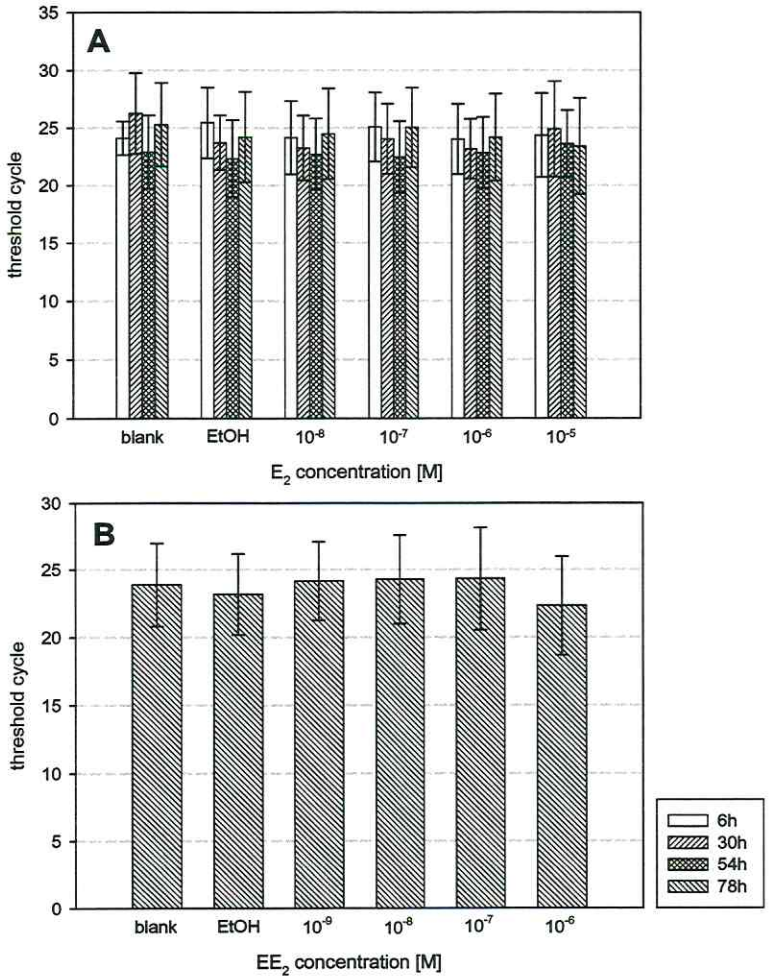

Figure 3.2.2 Mean threshold cycle of β-actin in qPCR. Bars repesent means ± standard deviation of independent PCR well plates. Values for each independent PCR well plate for respectively blank, EtOH, E_2, and EE_2 were obtained by averaging three replicate values. **A** cells upon exposure to E_2 of two fish for all exposure periods, number of independent PCR well plates was 36. **B** cells upon exposure to EE_2 of two fish at 78 h exposure, number of independent PCR well plates was 14.

Vitellogenin (VTG) and Vitelline Envelope Protein genes (VEPs α, β, γ)

In the absence of E_2 or EE_2, relative VTG mRNA abundance was found to be at or below the level of detection achieved by the qPCR assay, with the level of detection being set as described in Chapter 2.8. Similarly, relative mRNA expression levels for VEPs α, β, γ were difficult to determine in the absence of E_2 or EE_2 although these basal levels were more often at or just above, rather than below, the level of detection.

After 6 h of exposure to E_2, relative mRNA expression levels clearly rose above the level of detection for all four genes and with little discernible differences between concentrations. In fact, no differences in mRNA levels were observed for 10^{-8} to 10^{-5} M E_2 throughout the 78 h of exposure for either of the genes (Figure 3.2.3). The magnitude of the mean normalized expression was, however, found to differ between the genes with highest mRNA levels generally occurring for VTG. Thus, VTG mRNA expression was found to be approximately 10-fold higher than the expression for VEPs α, β, γ. This was true despite varying maximal mRNA levels in hepatocytes isolated from different fish. As shown in Figure 3.2.3, maximal levels varied by a factor of approximately 10 for all four genes in two independent experiments. Differences between the two experiments were also noted with regard to the time course of induction for the different genes. For VTG, mRNA abundance rose about 38-fold (Figure 3.2.3 A) and 14-fold (Figure 3.2.3 B) between 6 and 30 h of exposure with a slight further increase up to 54 h. For VEPs α, β, γ, a significant increase in mRNA abundance was observed up to 54 h of exposure in one experiment (Figure 3.2.3 A) but not in the other (Figure 3.2.3 B). These results overall indicated that VTG is a more robust indicator of estrogenic response but that the VEPs might be regulated more sensitively especially at the onset of the response.

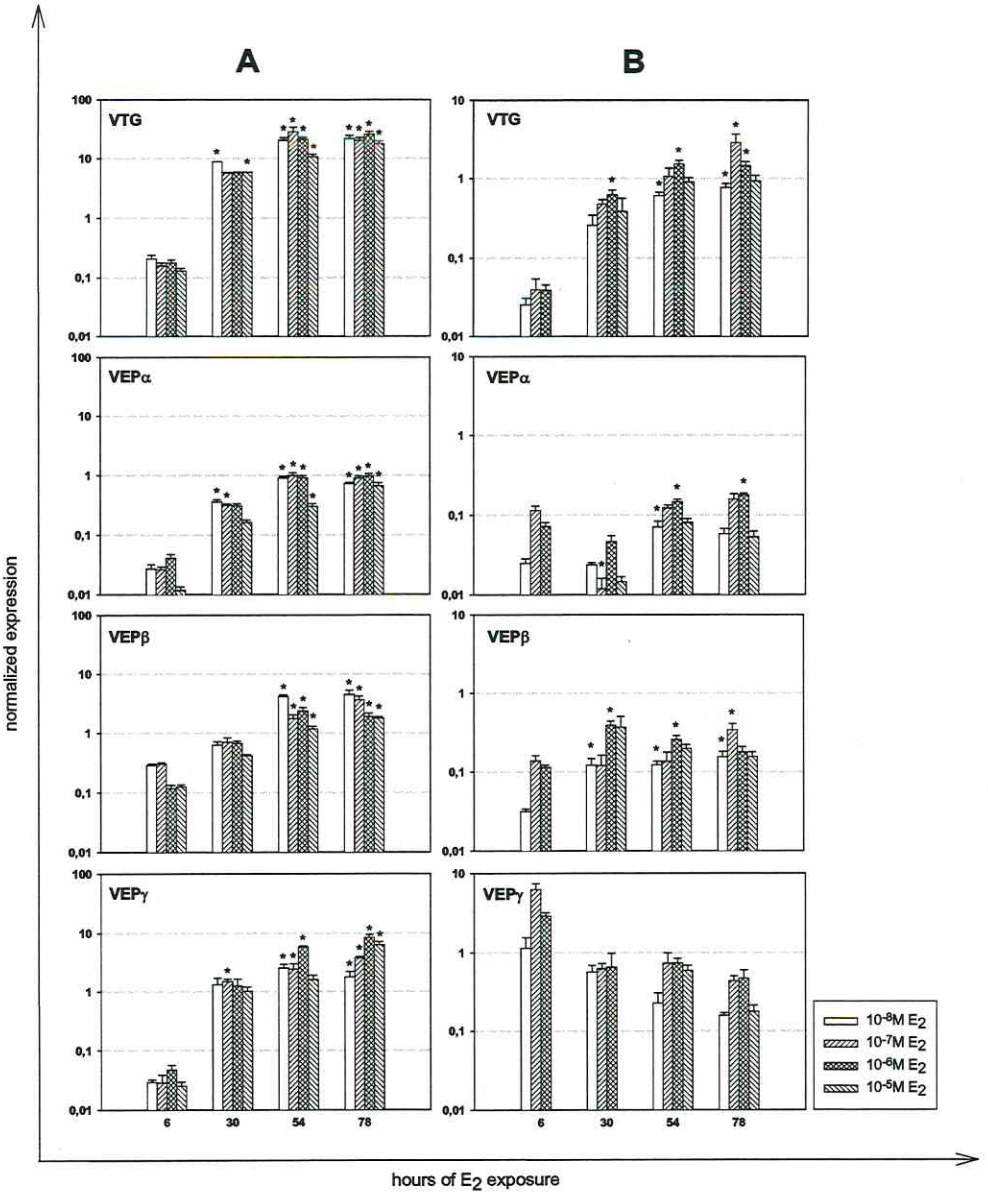

Figure 3.2.3 Time courses of induction of VTG and the VEPs α, β, γ mRNA abundance after treatment of primary hepatocytes from two independent experiments (**A** and **B**) with four concentrations of E_2. Each bar represents the mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2. Basal levels were below the level of detection. Asterisks indicate significant differences to the values at time point 6 h (ANOVA followed by Dunnett test, $P<0.05$). The maximal normalized expression level was 10-fold higher in **A** than in **B**. Missing values in **B** are due to insufficient RNA amounts derived from the cell cultures (10^{-5} M E_2 at 6 h) or due to degraded cDNA and insufficient raw material (RNA) for repeated analysis (10^{-5} M E_2 at 30 h for VEPγ).

In as much as concentrations of E_2 of 10^{-8} to 10^{-5} M all raised VTG and VEPs to approximately the same extent, four additional concentrations were subsequently tested for their ability to significantly induce VTG and VEP α, β, γ mRNA after 78 h of exposure. Among the four tested genes, only VTG and VEPα mRNA were significantly induced at 10^{-10} to 10^{-9} M E_2 with the level of expression for VTG being more than 10-fold higher than for VEPα (Figure 3.2.4). Appreciable levels of mRNA could not be observed for VEPβ and VEPγ upon exposure of hepatocytes to less than 10^{-8} M E_2 for 78 h.

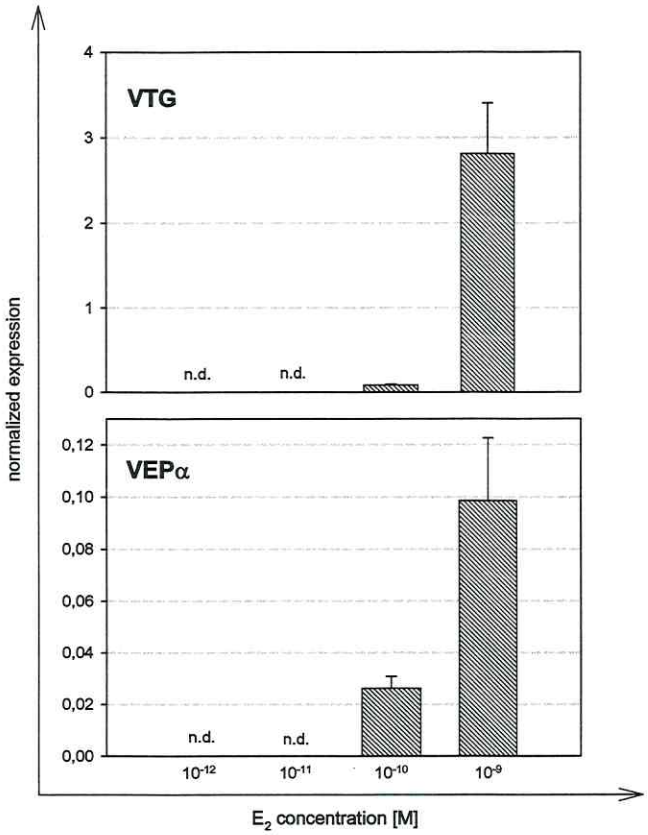

Figure 3.2.4 Induction of VTG and VEPα mRNA abundance upon exposure of hepatocytes from one independent experiment for 78 h to four concentrations of E_2. Each bar represents the mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2 n.d. = not detectable.

In addition to E_2, EE_2 was confirmed to significantly induce VTG and VEPs α, β, γ. Exposures focussed on 78 h and 10^{-9} M EE_2 was included as the lowest dose (Figure 3.2.5). Little difference in the expression levels were found for all four genes with regard to dose. Concerning expression levels, highest values were consistently obtained for VTG. While in the experiment shown in Figure 3.2.5, EE_2 induced VTG to maximal normalized expression levels of about 1, these were 6 in another experiment (data not shown). For the VEPs β and γ similar differences were observed in hepatocytes of two fish, while VEPα could be detected only in the fish represented in Figure 3.2.5. The difference in maximal relative expression levels for VTG and VEPs β, γ again confirms that these levels can vary between hepatocytes from different fish.

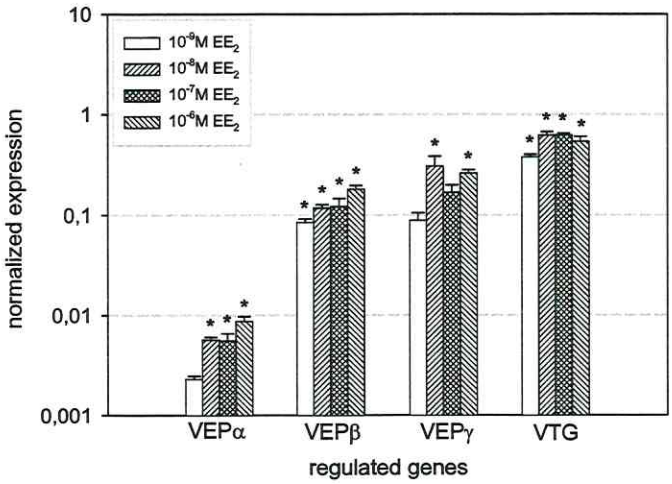

Figure 3.2.5 Induction of VTG and the VEPs α, β, γ mRNA abundance upon exposure of hepatocytes from one (of two) independent experiments for 78 h to four concentrations of EE_2. Each bar represents the mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2 Asterisks indicate significant differences to the solvent control (ANOVA followed by Dunnett test, $P<0.05$).

A co-exposure of E_2 or EE_2 treated cells with ICI 182,780 significantly diminished VTG and VEP α, β, γ mRNA abundance in the hepatocytes. As illustrated in Figure 3.2.6 for EE_2 exposed cells, inhibition generally occurred in a concentration-dependent manner for all four genes. Inhibition was greatest at 10^{-6} M ICI 182,780 although an appreciable reduction in mRNA abundance was observed for all four genes with as little as 10^{-8} M ICI 182,780, the lowest inhibitor concentration applied in this study. The regulation of expression of VEP γ and VTG appeared to be more susceptible to the inhibitory effect of ICI 182,780 than VEPs α and β.

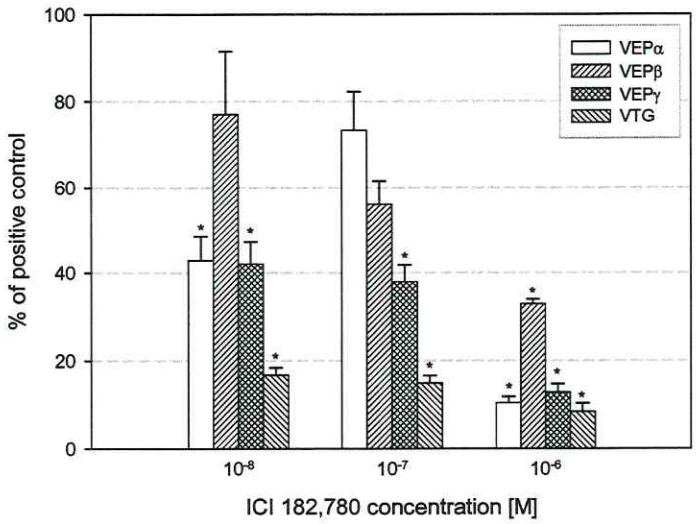

Figure 3.2.6 Inhibition of induction of VTG and the VEPs α, β, γ mRNA abundance after co-exposure for 78 h to 10^{-7} M EE_2 and various concentrations of ICI 182,780 from one (of two) independent experiments. Each bar represents the mean ± standard error of triplicates in percent relative to the positive control (hepatocytes exposed to 10^{-7} M EE_2 alone). Asterisks indicate significant differences to the positive control (unpaired one-tailed t test, $P<0.05$).

Serum albumin

In the absence of E_2 or EE_2, initial normalized gene expression for serum albumin varied between 3 and 50 units for four independent fish after 24 h in culture. Relative RNA expression levels significantly declined over the course of each experiment with less than 20 % remaining after 78 h of exposure (96 h of cell culture) (Figure 3.2.7). Based on this decline, the time to reach half the initial mRNA abundance ($t_{1/2}$) was determined to be 20±9 h (N=4 fish) and 27±8 h (N=4 fish) in blank and ethanol treated cells isolated from four independent experiments, respectively. As illustrated in Figure 3.2.7, the decline tended to be biphasic with a steep decline over the first days of culture and a moderate decline toward the end of the culture period.

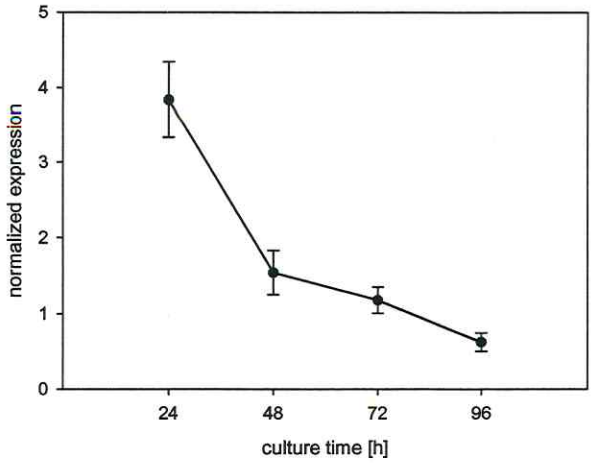

Figure 3.2.7 Reduction of the basal level of serum albumin mRNA abundance in one primary hepatocyte monolayer culture over 96 h. Data are given as mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2

The decline in basal mRNA abundance made observing E_2 or EE_2 related changes difficult. Nevertheless, a considerable decline in normalized mRNA expression compared to the ethanol control was observed upon 78 h of exposure to 10^{-5} M E_2. In two independent experiments, this decline was 55 % and 35 % (data not shown). In contrast to E_2, the presence of EE_2 in the medium appeared to ameliorate relative serum albumin mRNA expression levels after 78 h exposure in a concentration-dependent manner (Figure 3.2.8). This effect of EE_2 was not influenced by a co-exposure of the cells to ICI 182,780 (data not shown). Overall, the changes in relative mRNA expression levels for serum albumin did not exceed one order of magnitude for either the down-regulation during cell culture with or without E_2 or for the amelioration of down-regulation by EE_2.

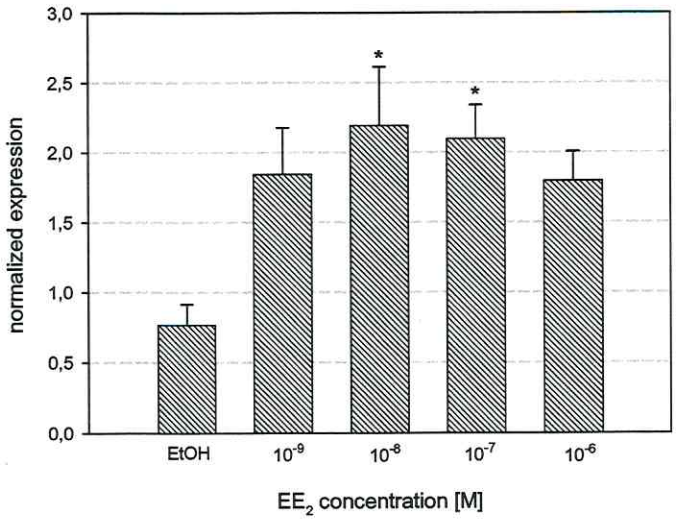

Figure 3.2.8 Concentration-dependent induction of serum albumin mRNA abundance upon exposure of hepatocytes from one (of two) independent experiment to four concentrations of EE_2 for 78 h. Values represent mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2 Asterisks indicate significant differences to the solvent control (ANOVA followed by Dunnett test, $P<0.05$).

Transferrin

In the absence of E_2 or EE_2, normalized gene expression for transferrin varied from 0.8 to approximately 10 units between hepatocyte isolations from different fish. Within one independent experiment, expression levels varied by a factor of up to two between different time points but a time-dependent effect could not be observed (data not shown). Upon 96 h in culture, normalized gene expression was 5.5±2.9 (N=5 fish) and 3.3±2.7 (N=5 fish) units for blank and solvent control, respectively.

Exposure of cells to E_2 led to a significant decline of relative transferrin mRNA expression levels (Figure 3.2.9) but not distinctly concentration dependent. However, a significant influence of time was detected (two way ANOVA). In contrast, no decline was observed upon exposure of hepatocytes to EE_2 (data not shown). Co-exposure of cells to ICI 182,780 did not appear to impact on the relative expression levels observed with E_2 or EE_2.

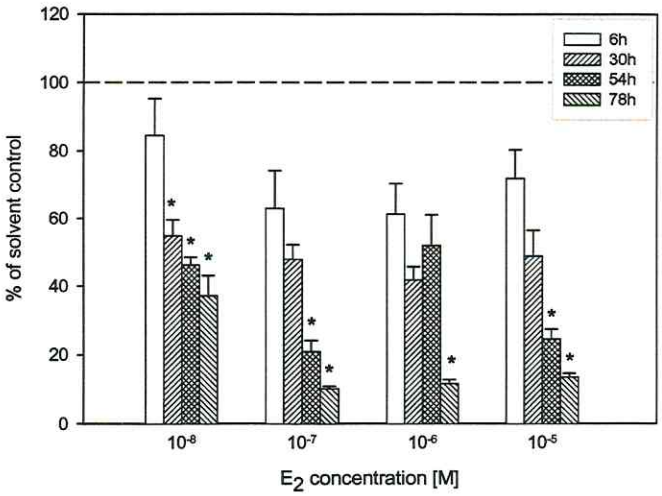

Figure 3.2.9 Time course of reduction of transferrin mRNA abundance after treatment of primary hepatocytes from one independent experiment with four concentrations of E_2. Each bar represents the mean ± standard error of triplicates in the qPCR in percent relative to the solvent control. Reference line represents the solvent control. Asterisks indicate significant reduction compared to the 6 h value for each concentration (ANOVA followed by Dunnett test, $P<0.05$).

CYP3A27

In the absence of E_2 or EE_2, normalized CYP3A27 gene expression was between 0.006 and 0.5 units for the blanks as well as the solvent controls with no significant deviations between the two. For example, after 96 h in culture and considering hepatocytes from eight independent experiments, average mRNA expression levels were 0.11±0.08 (N=8 fish) and 0.14±0.12 (N=8 fish) for blank and ethanol control, respectively. If constitutive expression values were compared over time, differences were occasionally observed but the only consistent pattern identifiable was a drop in mRNA abundance between the 24 and the 48 h time points. In five independent experiments (N=5 fish), this drop was found to be 13, 25, 55, 59 and 92%, with the latter three being significant. Beyond 48 h of cell culture, CYP3A27 mRNA abundance showed little further changes with no dependence on time.

Exposure of hepatocytes to varying concentrations of E_2 led to a significant time- and concentration-dependent reduction of relative CYP3A27 mRNA expression levels (Figure 3.2.10 A). Based on a two way ANOVA, a significant interaction between time and concentration was found. It can therefore not clearly be deduced, which of the factors has the greater influence on mRNA abundance. In a replicate experiment, focussing on the 78 h of exposure to E_2, the concentration-dependence of the decline in CYP3A27 mRNA was confirmed. Exposure to EE_2, which again focussed on the 78 h time point, also yielded a significant

concentration-dependent decline (Figure 3.2.10 B). The decline in mRNA abundance was abolished by co-exposure of cells to ICI 182,780 (Figure 3.2.11).

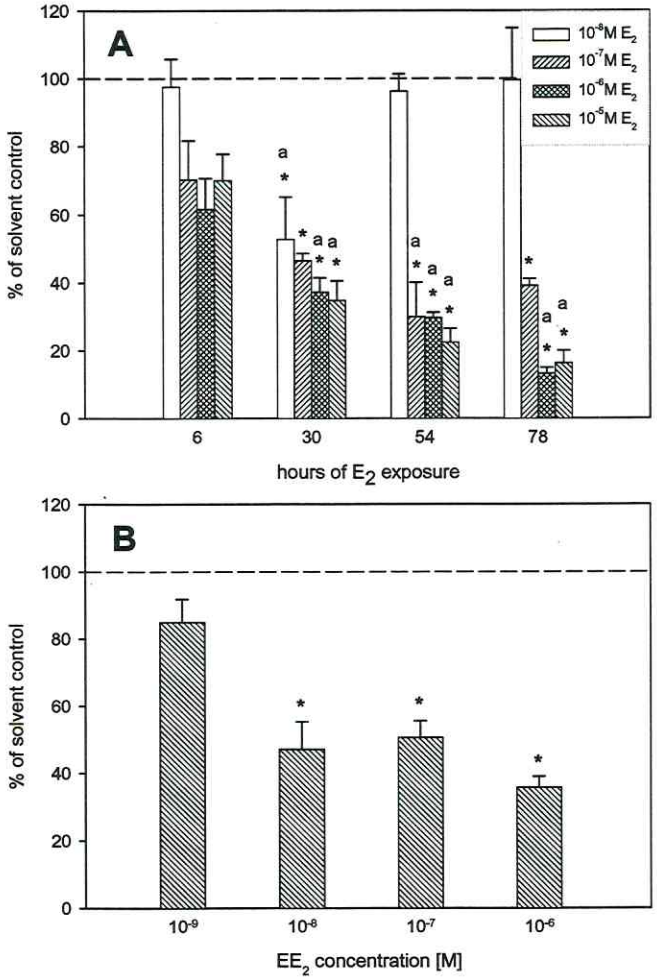

Figure 3.2.10 Changes of CYP3A27 mRNA abundance upon time and/or concentration-dependent exposures to E_2 (**A**) and EE_2 (**B**). Each bar represents the mean ± standard error of triplicates in the qPCR in percent relative to the solvent control. Reference line represents the solvent control (basal level of CYP3A27). Characters indicate significant reduction compared to the 6 h value for each concentration and asterisks indicate significant differences to the solvent control (ANOVA followed by Dunnett test, $P<0.05$). **A** Time course of reduction of CYP3A27 mRNA abundance after treatment of primary hepatocytes from one independent experiment with four concentrations of E_2. **B** Concentration-dependent decrease of CYP3A27 mRNA abundance upon exposure of hepatocytes from one (of two) independent isolations to four concentrations of EE_2 for 78 h.

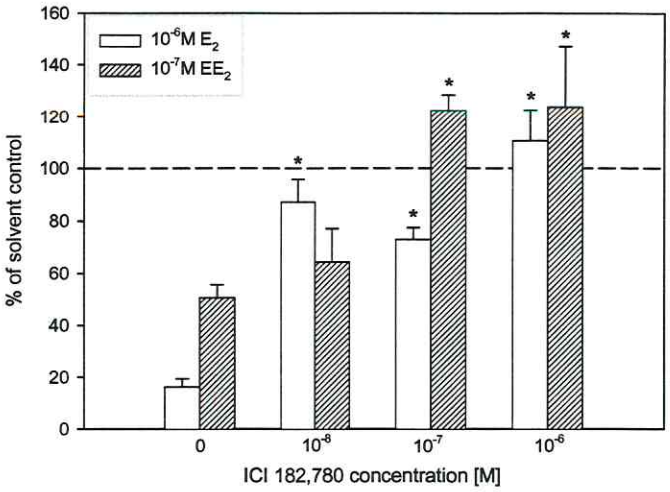

Figure 3.2.11 Levels of CYP3A27 mRNA abundance after co-exposure for 78 h to 10^{-7} M EE_2 (one of two independent experiments) or 10^{-6} M E_2 (one independent experiment) and various concentrations of ICI 182,780. Each bar represents the mean ± standard error of triplicates in percent relative to the solvent control. Reference line represents the solvent control. Asterisks indicate significant differences to the value without ICI 182,780 (unpaired one-tailed t test, $P<0.05$).

Discussion

In vitro cellular models offer several advantages for deciphering mechanisms of ecotoxicant action on the cellular and molecular level. In this study the well-known ecotoxicant EE_2 and its natural homolog E_2 were investigated concerning their effects on the regulation of a set of selected genes. The primary rainbow trout hepatocyte cultures used in this study easily sustained a 5-day culture with no apparent damage to the cells. Cell viability was assessed using two fluorescent indicator dyes, alamarBlue and CFDA-AM, which previously had been used to identify a decline in fish cell viability due to ecotoxicants in continuous cell lines (Dayeh *et al*, 2002b; Okamura *et al*, 2002; Schirmer *et al*, 1998b). As well in Chapter 3.1 of this thesis the two fluorescent assays were applied for the first time in primary fish hepatocytes. The reliability of the two fluorescent indicator dyes being used together was seen again in the identification of a cytotoxic effect elicited in the hepatocytes by 10^{-5} M ICI 182,780. The fact that this decline in cell viability was identified with the CFDA-AM assay but not alamarBlue points at ICI 182,780 as potentially inhibiting esterase activity or interfering with the uptake of CFDA-AM by the cells. These effects did not impact on cellular metabolism in general as seen with alamarBlue. A similar contrasting behaviour for the two fluorescent cell viability assays has been documented for 2-ethyl phenanthrene (Dayeh *et al*, 2003). Previous reports on the impact of ICI 182,780 on cell viability are controversial. In human lens epithelial cells ICI 182,780 at 10^{-7} M protected cells from the cytotoxic effects of H_2O_2 (Wang *et al*, 2003). On the other hand, as little as 10^{-11} M ICI 182,780 led to an inhibition of proliferation and an increase in apoptosis

in human umbilical vein endothelial cells (Soares *et al*, 2003). These diverse effects of ICI 182,780 need to be kept in mind when applying this estrogen-receptor antagonist for attempting to distinguish estrogen receptor-dependent from independent ecotoxicant effects. However, previous applications both in a fish cell line (Hornung *et al*, 2003) as well as in primary fish hepatocytes (Celius *et al*, 1999; Flouriot *et al*, 1995; 1996; Navas & Segner, 2001), along with the current work, are proof of the utility of ICI 182,780 or similar estrogen-receptor antagonists in mechanistic studies.

For the primary hepatocyte culture model, the β-actin gene was established as a valid house-keeping gene. Levels of β-actin mRNA were affected neither by the culture nor by the exposure conditions. This is in contrast to the pattern of β-actin gene expression observed in a suspension culture of primary rainbow trout hepatocytes, where mRNA levels increased over the first three culture days (Flouriot *et al*, 1993). Thus, basal levels of gene expression need to be carefully monitored for each gene and culture condition.

Although β-actin mRNA abundance across exposure conditions was found to be stable, differences were noted between individual hepatocyte isolations. These differences could potentially be due to experimental variation in cDNA syntheses or PCR or variation between fish. Ackermann (2000) found that the independent PCR reactions have more impact on experimental variation than independent cDNA syntheses. Along with the inter-PCR-variation of 4.1% in the present study, the greater differences observed in the β-actin mRNA abundance are thus more likely due to differences between individual fish. This was confirmed by significant differences in the response of the target genes in hepatocyte preparations of different fish, despite the similarity of expression patterns, which mirrors variations observed in whole animal studies. For instance, Buhler *et al.* (2000) found a testosterone CYP2M1 mRNA level induction in only two of four treated female rainbow trout. As well, differences in gene expression between animals were found by Ackermann (2002) and Hemmer *et al.* (2001). In fact, varying levels of mRNA abundance can even be found in cell lines (observation by Heidi Becker, Department of Cell Toxicology, UFZ). This variability due to individual differences hinders expression of results as mean normalized mRNA levels for independent fish. Expression of results in percent of solvent control is an alternative to deriving mean values between fish, but it represents a transformation step that masks true expression values. As well, if basal mRNA levels are not detectable in controls, a transformation into percent of control is not possible.

Basal normalized mRNA expression varied to a great extent between the target genes investigated here, ranging from non-detectable (generally below 10^{-2} to 10^{-3} normalized expression units) for VTG and VEPs α, β, γ to multiples of 10 units for the genes coding for serum albumin and transferrin. Both serum albumin and transferrin are crucial transport proteins whose synthesis in hepatocytes is one of the characteristic functions of the liver. The fact that albumin mRNA abundance significantly declined over the culture period is indicative of

a sensitive regulation of albumin mRNA abundance as hepatocytes adapt to the *in vitro* environment. An initial decline in albumin mRNA was previously found by Flouriot *et al.* (1993; 1998) in rainbow trout hepatocytes cultured in suspension. In these cultures, the decline of albumin mRNA was thought to correspond to the period of hepatocyte aggregation. In the current study, hepatocytes had formed aggregates within 20 h and after 48 h, very few single cells remained with little further changes to the aggregates. The 48 h time point corresponded to a slow-down in the decline of relative albumin mRNA expression levels. This supports the notion that serum albumin expression is down-regulated in hepatocytes mainly until the cell-to-cell contact is re-established.

Changes in basal mRNA abundance were not found for transferrin. Very few other studies have explored the time-dependent expression of transferrin in primary hepatocytes. According to Tamura *et al.* (1996), rat hepatocyte transferrin mRNA abundance did not decline over the first five days of monolayer culture and protein secretion was stable after an initial decline on the first day. Furthermore, monolayers of primary chicken hepatocytes were found to stably secret transferrin into the medium over the first six culture days while albumin secretion decreased (Fujii *et al*, 1996). Thus, basal expression of transferrin generally appears less susceptible than albumin to the changes occurring during the establishment of *in vitro* hepatocyte cultures. To identify whether this finding holds true, beyond the mRNA level, for protein synthesis and secretion in primary rainbow trout hepatocytes, is an important future task.

Transferrin and albumin appeared to be differentially regulated by exposure of cells to E_2 or EE_2. For albumin, an E_2-dependent decline in mRNA abundance was repeatedly observed after 78 h of exposure but only to the highest concentration applied, 10^{-5} M. This finding is in line with investigations by Flouriot *et al.* (1998) who identified 10^{-6} M E_2 as the cause of a rapid decrease in albumin gene transcriptional activity in suspension cultures of rainbow trout hepatocytes. No other study has investigated the influence of EE_2 on albumin expression and the mechanism of EE_2-elicited albumin mRNA augmentation remains elusive at this point. Yet, the estrogen receptor does not seem to be involved. Despite these observed E_2/EE_2-dependent regulations, the albumin gene represents an example of a gene whose regulation should not preferentially be studied in a short-term culture system such as the five-day monolayer culture, where stable background levels cannot be ascertained. A long-term culture system, such as the one applied by Flouriot *et al.* (1993; 1998), seems a more appropriate alternative in this case.

Exposure of cells to E_2 significantly affected transferrin mRNA abundance whereas exposure to EE_2 did not. Upon water exposures to sheepshead minnows, E_2 (65 ng/L) was found to more strongly reduce transferrin mRNA expression levels than EE_2 (109 ng/L) (Larkin *et al*, 2003a). In *Xenopus* liver *in vivo* and *in vitro*, mRNA transcripts accounting for 85% of the serum proteins constitutively synthesized (including transferrin and albumin) were generally found to be down-regulated by E_2, with the main mechanism being mRNA destabilization (Pastori *et al*, 1991). This mechanism, however, does not play a role in salmonids where an overall competition

between VTG and other E_2-inducible genes for RNA polymerase, transcription factors or translational machinery was postulated as the general cause underlying a serum protein mRNA decline (Flouriot *et al*, 1998). In the current study, EE_2 was found ineffective in regulating transferrin mRNA levels at concentrations eliciting significant induction of VTG. As well, a significant abolishment of E_2-elicited reduction in transferrin mRNA abundance could not be ascertained using the estrogen receptor antagonist ICI 182,780. Thus, the regulation of liver-derived serum protein genes in general, and of transferrin specifically, appears to also involve factors that are independent of the estrogen receptor and the induction of VTG synthesis.

Apart from serum albumin and transferrin as general indicators of hepatocyte differentiated function, E_2-induced VTG synthesis has been suggested as a marker for rainbow trout primary hepatocyte differentiation (Flouriot *et al*, 1993; Maitre *et al*, 1986; Vaillant *et al*, 1988). Pelissero *et al.* (1993) and Jobling & Sumpter (1993) were the first to suggest the use of VTG induction in rainbow trout hepatocytes to detect ecotoxicants with estrogenic activity. VTG mRNA abundance was significantly induced in the current work by both E_2 and EE_2 and inhibition of induction due to ICI 182,780 confirmed the role of the estrogen receptor in this cellular response. Induction of normalized mRNA expression was supported by a significant accumulation of VTG protein in the culture medium as determined by ELISA (data not shown). Taken together, the induction of VTG underlines the differentiated state of the primary hepatocyte cultures and confirms the applicability of gene expression *in vitro* for detecting estrogenic effects.

Concerning the dynamic range and the role of exposure time, mRNA abundance in the hepatocytes was comparable to the induction described for female juvenile rainbow trout exposed to E_2-spiked laboratory water (Thomas-Jones *et al*, 2003). Whether mRNA abundance was also comparable in terms of E_2 concentrations is difficult to state. In Thomas-Jones *et al.* (2003) concentrations in water were in the range of 2.3 ng/L (8.5×10^{-12} M) to 463 ng/L (1.7×10^{-9} M). In the *in vitro* experiments described here, significant induction of at least VTG was found between nominal E_2 concentrations of 10^{-10} M (27 ng/L) and 10^{-9} M (270 ng/L). In the plasma of vitellogenic female rainbow trout, the serum E_2 level was reported to amount to about 20 µg/L (Jobling, 1995). A direct comparison of an *in vitro* and *in vivo* effective concentration would require knowledge of the truly available ecotoxicant concentration at the target site. Thus far, these concentrations have rarely been determined *in vitro* and would be very difficult to obtain *in vivo*.

The ecotoxicant concentrations used in the current study were chosen based on previous studies focussing on the well-established marker VTG in primary hepatocytes (Navas & Segner, 2000; Hwang *et al*, 2000). In these studies, nominal concentrations of 10^{-8} M and 10^{-7} M were found to maximally induce a VTG response at the level of gene or protein expression. A maximal induction at these concentrations was confirmed here on the gene expression level. Another two higher concentrations, 10^{-6} M and 10^{-5} M, were added in order to enable the

detection of responses of genes which had not yet been studied in the hepatocyte model. For instance the decrease of CYP3A27 mRNA abundance was found at E_2 concentrations of 10^{-7} M and higher (Figure 3.2.10 A). All these concentrations are nominal concentrations which cannot distinguish the freely accessible amount. It is assumed that the free concentrations of E_2 and EE_2 are appreciably lower than the used nominal because of sorption, e.g. to the tissue culture well walls. Along these lines, Longman & Buehring (1986) found a loss of freely available E_2 of about 38 % in 24 well polystyrene plates due to sorption. Currently, more efforts are made for developing techniques to determine the free concentrations of the target substance in the test vessel (i.e. Heringa 2004) or even within the cells (Schirmer *et al*, 1997; Bopp, 2004). In the future, it would be an important component to determine the amount of the tested compound remaining in the cell culture medium or even better directly within the cells.

Along with VTG, VEPs α, β, γ mRNAs were significantly induced upon E_2 or EE_2 exposure, and this induction could again be blocked by a co-treatment with ICI 182,780. It has been suggested previously that VEPs may, with respect to time and dose, be more sensitively induced by estrogens than VTG (Arukwe *et al*, 1997; Westerlund *et al*, 2001; Celius *et al*, 1999; 2000), although indifference in the expression of VEPs and VTG has also been described (Ackermann *et al*, 2002). In the current study, the normalized mRNA expression of VEPs α, β, γ overall appeared slightly more sensitive than VTG with regard to the onset of induction. However, VTG was at least as sensitive with regard to exposure concentrations and resulted in higher maximal mRNA levels than the VEPs. Nevertheless, differences among the VEPs have been observed that do agree with previous reports. For example, at the earliest time point in this study (6 h of E_2 exposure), the only VEP that consistently gave a large rise in normalized mRNA expression was VEP β. This observation is in agreement with Westerlund *et al.* (2001) who identified VEP β mRNA to be expressed more strongly than VEPs α and γ one hour after E_2 injection into arctic char. As well, the fact that VEP γ was most responsive to EE_2 exposure in the primary hepatocytes is a confirmation of the finding described for EE_2-exposed female rainbow trout (Thomas-Jones *et al*, 2003). The co-exposure of hepatocytes with the estrogen inhibitor ICI 182,780 yielded another interesting difference between VEPs and VTG: VTG induction was inhibited more strongly at lower inhibitor concentrations than the VEPs. These findings support the perception by Westerlund *et al.* (2001) that the mechanisms underlying the regulation of VEP expression in teleost fish is not yet fully understood and that more in-depth investigations are needed prior to implementing VEPs as early biomarkers of endocrine disruption.

Beyond VTG and VEPs α, β, γ, CYP3A27 constitutive gene expression was regulated by both E_2 and EE_2 and this regulation was abolished by co-exposure with ICI 182,780. Previously, E_2 had been shown to diminish CYP3A27 mRNA and protein levels as well as the CYP3A27-dependent progesterone 6β-hydroxylase enzyme activity in rainbow trout injected with E_2 for 19 days (Buhler *et al*, 2000). Further, EE_2 was discussed as a potential specific inhibitor of CYP3A27, based on results obtained in trout liver microsomes (Miranda *et al*, 1998). In fact, E_2 has been

identified to suppress basal levels, both on the pre- as well as on the posttranscriptional level, of several hepatic cytochrome P450s in rainbow trout, including CYP2M1, CYP2K1 (Buhler *et al*, 2000) and CYP1A (Navas & Segner, 2001). In the latter study on CYP1A, which used primary rainbow trout hepatocytes, inhibition by E_2 was shown to be eradicated by the estrogen receptor antagonist Tamoxifen. The results presented here indicate for the first time that constitutive CYP3A27 is regulated as well, at least in part, on the transcriptional level upon involvement of the estrogen receptor. Future studies to explore these mechanisms should benefit greatly from the primary hepatocyte *in vitro* model.

Taken together, it was shown that in the primary rainbow trout hepatocyte model, VTG overall is a more robust marker of an estrogenic response than the VEPs but that further research is needed to understand the regulation of VEP gene expression. The cytochrome CYP3A27, a homolog to the human CYP3A4 playing a significant role in the metabolism of pharmaceuticals in human liver, was identified for the first time to be under estrogen-receptor control. Critical parameters of the *in vitro* assay are the monitoring of basal levels of gene expression during the culture period and the variation in expression levels between hepatocytes isolated from different fish. With respect to mechanistic ecotoxicology, the selected-gene assessment approach appears particularly useful for exploring suspected cellular targets of pharmaceuticals, such as clofibric acid, in non-target species, such as fish (Chapter 3.3). On the other hand, parallel gene expression analysis tools, such as DNA microarrays (Chapter 3.4), could be applied to the hepatocyte cultures in combination with the qPCR in order to identify previously unknown genes. Thus, the combined *in vitro*/molecular approach can function as a key to derive relevant mechanistic parameters for effect assessment based on causality.

Consequences for Chapter 3.3 and Chapter 3.4

The work presented in Chapter 3.2 yielded four important conclusions for the further development of this thesis. Firstly, the combined alamarBlue/CFDA-AM assay introduced in Chapter 3.1 proved to be very useful for the determination of acutely toxic concentrations of the substance tested. It provides an easy and valuable tool for the fast pre-screening of ecotoxicants such as clofibric acid in Chapter 3.3. Secondly, β-actin was established as a robust house-keeping gene in terms of hepatocyte culturing and exposure to E_2 and EE_2. Thus, this gene was further proposed as a house-keeping gene in the remaining chapters of this thesis. Thirdly, the evaluation of the responses of the four estrogen regulated genes VTG, VEPs α, β, γ, showed that VTG seemed to be the most robust biomarker. It is for this reason that the investigations on clofibric acid in Chapter 3.3 focussed on VTG gene expression for detecting estrogenic effects. Finally, the time- and concentration dependent studies described in this chapter facilitated the decision about concentration and exposure time for the cDNA-array experiments: The chosen conditions were 10^{-7} M E_2 and 10^{-7} M EE_2 for 54 h (Chapter 3.4).

Chapter 3.3

Effects of clofibric acid - a pharmaceutical of environmental concern - on gene expression

Introduction

Currently, pharmaceuticals attract attention in many ways, not only in terms of spectacular prosperities in human medicine. Also policy makers are paying attention to them as they are creating new laws like the EU chemicals legislation - REACH (Registration, Evaluation, Authorisation and Restriction of CHemicals) in order to meet new requirements concerning environmental and human health. Here, the assessment of ecotoxicological risks plays an important role in environmental protection.

One major source of pharmaceuticals is human medicine. In general, the active components of pharmaceuticals are excreted almost unmodified. Even in sewage treatment plants little biological degradation takes place for some of the pharmaceuticals. Due to these facts, pharmaceuticals are continuously introduced into surface water via sewage effluents (Stumpf *et al*, 1996; Ternes *et al*, 1999; 2002).

This study focussed on clofibric acid (CA), the active substance of lipid lowering drugs (e.g. clofibrate, etofyllin clofibrate, etofibrate), which are used in human medical care. It is prescribed to counteract high amounts of cholesterol and triglyceride in blood. Recent studies on the mode of action of CA identified it as a specific ligand for the peroxisome proliferator activated receptor α (PPARα) (Kliewer *et al*, 1997; Forman *et al*, 1997; Staels *et al*, 1997). This receptor is a member of the nuclear hormone receptor superfamily and is localized in the nucleus (Dreyer *et al*, 1992). Fatty acids and eicosanoids have been identified to be the natural ligands for PPARα (Forman *et al*, 1997). After activation of the receptor by its respective ligand, it forms a heterodimer with the receptor for 9*cis*-retinoic acid (RXR) and binds to specific responsive elements in the regulatory region of target genes (Figure 3.3.1). One of the induced target genes is that coding for the enzyme lipoprotein lipase (LPL), which is involved in fatty acid metabolism in the blood. The increased synthesis of lipoprotein lipase in the liver benefits the lipoprotein lipolysis and therefore enhances lipid catabolism.

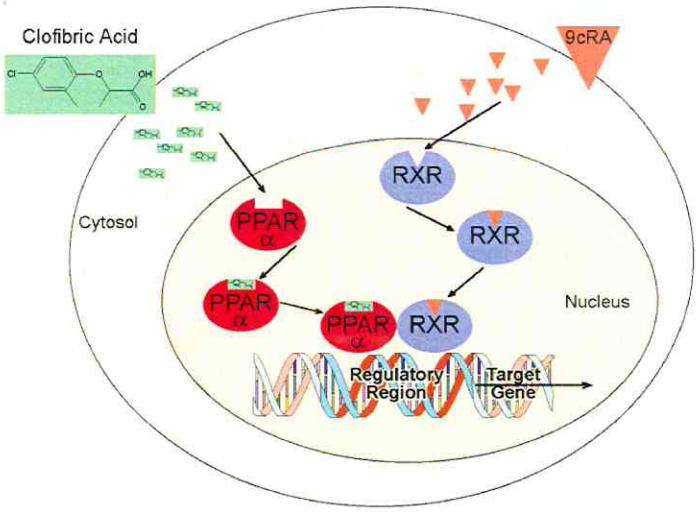

Figure 3.3.1 Signalling pathway of PPARα by its synthetic ligand clofibric acid. For detailed explanation, see text above.

In the early nineties of the last century CA was detected for the first time in the environment in Germany. Groundwater in Berlin contained up to 300 ng/L ($1{,}5x10^{-9}$ M) CA in 1992 (Stan & Linkerhägner, 1992). This alarming finding led to extensive investigations of pharmaceutical contents in surface-, ground and tap-water in Germany. CA was detected in surface waters in Hessen up to 180 ng/L ($0{,}9x10^{-9}$ M) (Stumpf *et al*, 1996). In the year 2002, CA concentrations of up to 7.3 µg/L ($3{,}7x10^{-8}$ M) were found in Berlin's groundwater (Heberer, 2002). Even in tap-water, 40 ng/L ($2x10^{-10}$ M) CA (in Berlin), and <100 ng/L (<$5x10^{-10}$ M) (in Hessen) were found (Heberer, 2002; Stumpf *et al*, 1996). In contrast, measurements in the city and surrounding area of Cologne yielded no or very low CA concentrations (Jux *et al*, 2002).

The persistency of most of the pharmaceuticals (including CA) explains this accumulation in the environment. The sewage treatment plants are not able to remove all of these compounds. For instance, no removal of CA could be detected in three sewage treatment plants in Berlin (Heberer, 2002). However, in a municipal sewage treatment plant near Frankfurt/Main a removal rate of 51% of CA was observed. Nevertheless, the effluent still contained 1.6 µg/L ($8x10^{-9}$ M) CA (Ternes, 1998). Currently, efforts have been made to enhance the removal of pharmaceuticals during their passage through sewage treatment plants. Among these investigations, CA has proven to be very persistent (Ternes *et al*, 2002; Zwiener & Frimmel, 2003).

All these facts together cause concern about the potential chronic effects of CA not only in humans (as indicated by detected concentrations in tap-water) but also in non-target, exposed species like fish living in rivers downstream of sewage treatment plants. Only few ecotoxicological data are available for pharmaceuticals in general. Up to now three publications

present experimental ecotoxicological data for clofibric acid (Henschel *et al*, 1997; Cleuvers, 2003; Ferrari *et al*, 2003). All of them were restricted to standard toxicity tests with living organisms (algae, bacteria, zebra fish embryo, duckweed) except for one. Henschel and colleages (1997) extended the array of tested models by the fish cell line BF-2 from bluegill sunfish using inhibition of proliferation as endpoint with a determined EC_{50} value of 14 mg/L ($7x10^{-5}$ M).

The present study aims at improving ecotoxicological effect assessment of the pharmaceutically active substance clofibric acid, which is widespread in the aquatic environment. Primary hepatocytes from rainbow trout as *in vitro* model were used to examine whether CA is cytotoxic and whether CA can act as a PPARα ligand in liver cells of a non-target species by inducing a PPARα-activated gene. As indicator of the successful interaction with PPARα, the mRNA abundance of lipoprotein lipase (LPL) after exposure to CA was determined with real-time rt-PCR (qPCR). As well, vitellogenin (VTG) was included as a marker of estrogenic effects because continuously treated human male were shown to undergo a slight feminisation (The Coronary Drug Project Research Group, 1975). Co-exposures to CA and the estrogen receptor blocker ICI 182,780 were carried out to estimate the potential involvement of the estrogen receptor in the alteration of gene expression. In addition, four other genes were included in this study. These were β-actin, which was investigated as a house-keeping gene, serum albumin (SA) and transferrin as indicators of hepatocyte differentiated function and CYP3A27 as a member of the CYP3A family, which is involved in the metabolism of pharmaceuticals in humans.

Results

In the following, results of cell viability assays and gene expression studies from primary hepatocyte cultures exposed to CA are presented. Results of the complete time courses were obtained from three independent experiments with two female and one male rainbow trout. Co-exposures of CA with the estrogen receptor antagonist ICI 182,780 were done in hepatocytes of two additional female fish for the expression of LPL, CYP3A27, SA, transferrin and VTG. None of the experiments with ICI 182,780 showed any effect (data not shown). However, the studies on VTG expression in CA/ICI 182,780 co-exposures were hampered by a lack of endogenous VTG mRNA abundance in hepatocytes of the particular fish, which appeared to play a role in the impact of CA on VTG mRNA levels in female trout (see below).

Cell viability assays with alamarBlue and CFDA-AM

Primary hepatocyte cultures were monitored for cell viability over the course of the experiment at each time point immediately prior to RNA isolation, using the fluorescent indicator dyes alamarBlue, for metabolic activitiy, and CFDA-AM, for general membrane integrity (see also Chapter 3.1). As mentioned also in Chapter 3.2, consistent unit values in cells without

treatment (blank) as well as in the solvent control (EtOH) indicated no alteration in cell viability. In addition, the fluorescent units showed only little variation with increasing CA concentrations. In the alamarBlue assay, slight but significant increases in the fluorescent units were observed at the highest (10^{-5} M) CA concentration (Figure 3.3.2). In agreement with this observation, a significant influence of concentration was detected (two way ANOVA). In contrast, no significant alteration in cell viability was found in the CFDA-AM assay.

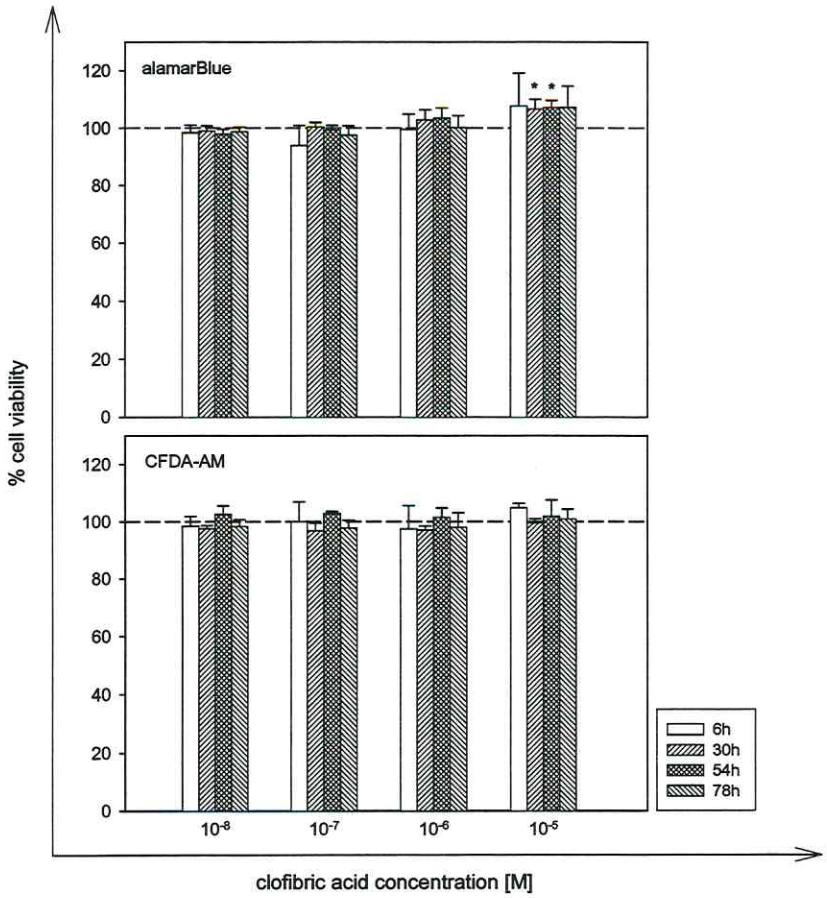

Figure 3.3.2 Effect of clofibric acid on cell viability assessed with the combined alamarBlue/CFDA-AM assay. Data are given as mean ± standard deviation (N=3 fish) in percent relative to the solvent control, the latter of which is indicated by the reference line at 100%. Asterisks indicate significant changes compared to the solvent control (one way ANOVA followed by Dunnett test, $P<0.05$)

Evaluation of the house-keeping gene β-actin

The house-keeping gene β-actin showed no significant alterations in mRNA abundance. Neither the treatment with four CA concentrations (Figure 3.3.3) nor the duration of culturing significantly influenced the mRNA amount of β-actin (data not shown).

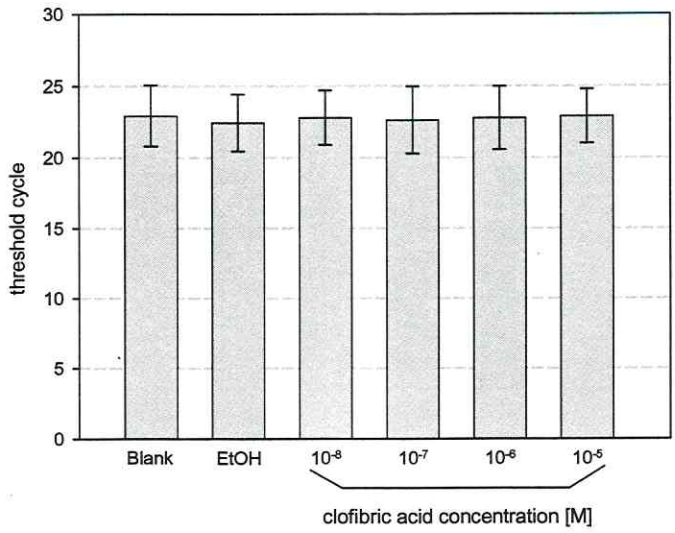

Figure 3.3.3 Mean threshold cycle of β-actin in qPCR upon exposure of cells to clofibric acid (N=3 fish). β-actin was measured in all samples of these 3 fish. Bars represent means ± standard deviation of n=23 means of triplicates.

Lipoprotein lipase

In the two independent experiments with hepatocytes from female trouts, lipoprotein lipase mRNA was detectable, but not in the hepatocytes from the male fish. A distinct pattern of the regulation of lipoprotein lipase mRNA expression for the two female rainbow trouts could not be derived (Figure 3.3.4). Even if a regulation were to occur it would have been very difficult to detect because the normalized mRNA expression was very low overall. It differed between 0.01 and 0.25 units in the two experiments including the solvent controls.

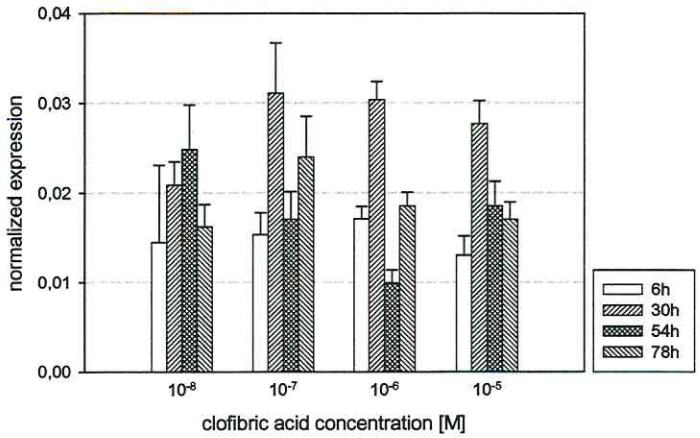

Figure 3.3.4 Time course of lipoprotein lipase mRNA abundance after treatment of hepatocytes from one female fish with four concentrations of CA. Each bar represents the mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2.

Vitellogenin

Exposure of hepatocyte cultures from one of the two females (Figure 3.3.5 A) and the male fish (Figure 3.3.5 B) to varying concentrations of CA, led to significant dose and time dependent alterations of VTG mRNA abundance. No VTG mRNA was detectable in hepatocytes of the second female. In the experiment with hepatocytes from a male fish, VTG mRNA expression levels could not be detected at the time points 6 h and 30 h of exposure. CA led to a significant time dependent increase of VTG at 54 h and 78 h of exposure of hepatocytes from the male fish except for the lowest concentration tested (10^{-8} M CA) (Figure 3.3.5 B). Based on two way ANOVA a significant interaction between concentration and time was found. Therefore, it could not be clearly deduced which parameter effects the increase of mRNA abundance to a higher extent.

The time course with hepatocytes isolated from a female trout showed detectable VTG mRNA abundance at all time points. In the absence of CA, a significant level of relative VTG mRNA was found at the beginning of the experiment, which indicates a beginning vitellogenesis. A significant decrease of VTG mRNA expression levels was found for the control as well as for the

CA exposed cells after 30 h of CA exposure compared to the 6 h time point. At the time points 30 h and 54 h the VTG mRNA abundance did not change. After 78 h of exposure, however, VTG mRNA abundance significantly increased for all CA exposed cells but not the control. The increase in VTG mRNA abundance was dose-dependent with levels of expression being similar to the 6 h time point at concentrations larger than 10^{-7} M of CA. Thus, an extremely significant influence of time was detected ($P<0.0001$) and additionally a significant interaction between concentration and time (two way ANOVA).

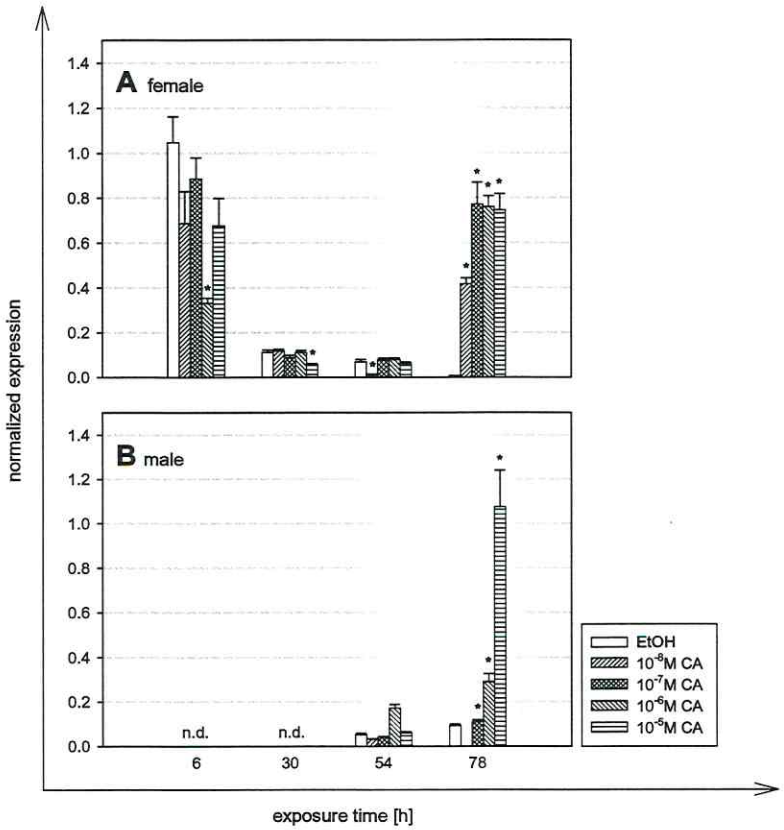

Figure 3.3.5 Time course of the levels of VTG mRNA abundance after treatment of primary hepatocytes with CA or the solvent control. Each bar represents the mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2. Results in graph **A** were obtained from an experiment with hepatocytes from an individual female fish. Asterisks indicate dose dependent differences to the solvent control (one way ANOVA followed by Dunnett test, $P<0.05$). Results in graph **B** were obtained from an experiment with hepatocytes from an individual male fish. At time points 6 h and 30 h no detectable mRNA abundance was observed. Asterisks in this case indicate significant differences over time compared to the values at time point 54h (unpaired t-test, $P<0.05$). n.d. = not detectable.

Because the two experiments in Figure 3.3.5 were indicative of an estrogenic response due to CA exposure, an experiment was conducted where cells were intentionally exposed to 10^{-9} M E_2. The VTG response on the mRNA level at this E_2 concentration is in the beginning, not in the maximum range (see Chapter 3.2). The idea was to simulate a vitellogenesis in the hepatocytes of the female trout to medium induction levels of VTG mRNA. These cultures were co-exposed to CA to see if CA could potentiate VTG induction as was indicated in the experiment in Figure 3.3.5 A. This effect was not observed. Co-exposure to 10^{-9} M E_2 and 10^{-6} M CA led to ~70% of the VTG response of 10^{-9} M E_2 alone (Figure 3.3.6).

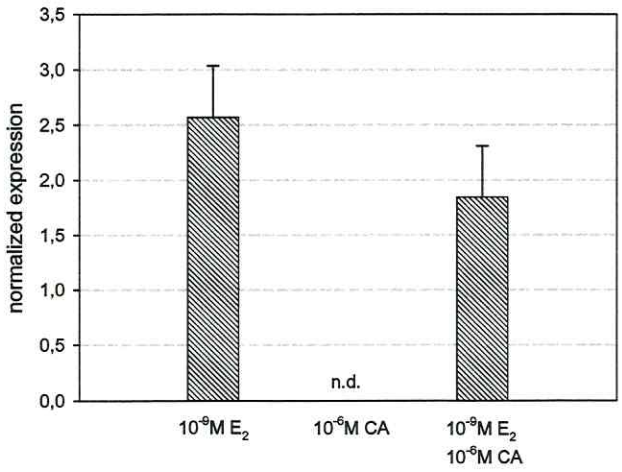

Figure 3.3.6 VTG mRNA abundance upon exposure of hepatocytes from one female trout to E_2, CA or a mixture of the two for 78 h. Each bar represents the mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2. n.d. = not detectable.

CYP3A27

The exposure of hepatocytes to four concentrations of CA led to no significant alterations in CYP3A27 relative mRNA expression levels either in male (Figure 3.3.7) or in the female fish. Overall the normalized mRNA expression level was in a very low range, varying between 0.004 and 0.27 units including the solvent controls.

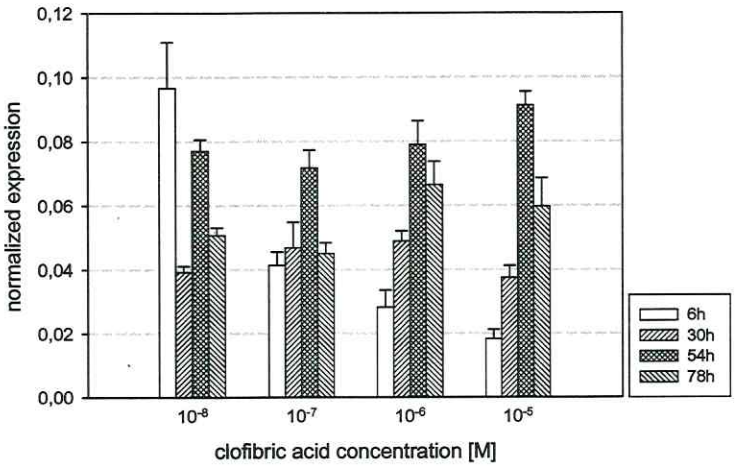

Figure 3.3.7 Time course of CYP3A27 mRNA abundance after treatment of hepatocytes from an individual male fish to four concentrations of CA. Each bar represents the mean ± standard error of triplicates in the qPCR as calculated according to Chapter 2.8.2.

Serum Albumin and Transferrin

As described in Chapter 3.2, serum albumin mRNA abundance significantly declined over time in untreated cells. Under these conditions, no consistent alterations in serum albumin mRNA expression levels upon CA exposure were found.

A complete time course of transferrin mRNA abundance was available only for the hepatocyte cultures from two female fish. The normalized mRNA expression levels were one order of magnitude higher in one female (between 2 and 10 units) than in the other (between 0.03 and 1.6 units). Overall, no clear tendency of a time or concentration dependent regulation of transferrin mRNA abundance was found.

Discussion

The persistent and ubiquitary appearance of a number of pharmaceuticals in the aquatic environment is of growing concern, particularly because of their potential effects on non-target but chronically exposed species like fish. Although some possible cellular targets may be deduced from knowledge gained in pharmaceutical research in mammals, the impact on non-target species should be difficult to predict overall. CA belongs to the category of peroxisome proliferators classified in rodents (Reddy & Lalwai, 1983). However, CA has no peroxisome proliferative effect in human hepatocytes (Green, 1995). In rainbow trout, two other members of the peroxisome proliferators (ciprofibrate and gemfibrozil) elicited peroxisome proliferation *in vivo* (Yang *et al*, 1990; Scarano *et al*, 1994). This indicates that a compound or similar compounds do not necessarily cause the same effect in different species, even not in all mammals.

In this study, the impact of clofibric acid on rainbow trout primary hepatocytes *in vitro* was investigated. The results of this study were derived from a total of four female (two for complete time courses and two for investigations with ICI 182,780) and one male rainbow trout. It would have been interesting to have added more male fish to these investigations. Yet, as the fish farm providing the trouts prefers female fish, it was impossible to repeat the experiment with a male: the ratio of male/female is 1:50.

The tested concentrations of CA caused no deleterious effect on cell viability of the primary hepatocytes. In the study of Qu *et al.* (2001), a significant loss of cell viability was found after 1 h treatment in the mouse hepatocyte cell line AML-12 with the MTT test but only at concentrations $\geq 0.9 \times 10^{-3}$ M (180 mg/L) clofibrate, the predrug of clofibric acid. This concentration is 90-fold higher than the highest concentration of CA used in this study (10^{-5} M). Henschel et al. (1997) found an EC_{50} value of 14 mg/L (7×10^{-5} M) in the inhibition of proliferation in the fish cell line BF-2. In contrast to Qu *et al.* (2001) and Henschel *et al.* (1997), an increase of the amount of the fluorescent product of alamarBlue was found at 10^{-5} M CA at all time points in the present study. This indicates that CA induces a mitochondrial proliferation in hepatocytes from rainbow trout because alamarBlue is mainly metabolized by enzymes located on the mitochondrial membrane (Gonzalez & Tarloff, 2001). This is in line with the study of Hoivik and colleagues (2004), who found a peroxisome and mitochondrial proliferative effect but no cellular proliferation in monkeys upon fibrate exposure. Inasmuch as peroxisomes and mitochondria can catabolize fatty acids via β-oxidation, their proliferation appears to be one means by which CA increases fatty acid metabolism.

For the primary hepatocyte culture model, β-actin was established as a valid house-keeping gene. Levels of β-actin mRNA were affected neither by the culture conditions nor the treatment with CA. For this reason, β-actin was used as the reference for normalizing mRNA levels of the genes of interest.

Using β-actin for normalizing gene expression data, transferrin and serum albumin did not appear to be regulated by exposure of hepatocytes to four concentrations of CA. As well, no alterations were found for mRNA levels of CYP3A27. Both, serum albumin and transferrin are general indicators of hepatocyte differentiated function. They code for crucial transport proteins, whose synthesis in hepatocytes is a characteristic function of the liver. These important functions are not affected at least on the level of gene expression by CA exposure in the applied concentration range. Likewise, CA does not appear to be a substrate of CYP3A27 or impact otherwise on its expression.

The therapeutic effect of clofibric acid in humans is, at least in part, mediated by the PPARα and the induced regulation of gene expression. This pharmaceutical induced the lipoprotein lipase gene transcription in the liver of humans and rodents (Staels *et al*, 1998). In the present study, no induction of LPL gene expression was found in hepatocytes from rainbow trout. The failed response of the LPL gene might be due to a lack of the receptor PPARα in primary rainbow trout hepatocytes, which could be tested in the future by e.g. designing primers for rainbow trout PPARα (a partial sequence is available in GenBank AY494835). Another explanation for the lack of an LPL response might be different binding properties of the PPARα receptor in rainbow trout.

Since a few years a link between lipid lowering drugs and estrogens is discussed: estrogens are more strongly metabolized in the presence of fibrates to estradiol fatty acid esters (Gonzalez, 2002). These esterified metabolites of estradiol have long half-lives *in vivo* (Hershcopf *et al*, 1985) and are present in fat-rich tissues. Thus, they serve as a prolonged source of steroid even though by themselves they show little ability to bind to the estrogen receptor (Larner *et al*, 1985; Zielinski *et al*, 1991). Only after the hydrolysis to the free steroid E_2, the expected receptor binding can be found (Janocko *et al*, 1984). However, male humans continuously receiving clofibrate showed side effects related to disturbed sex hormone function such as decreased libido and breast tenderness (The Coronary Drug Project Research Group, 1975). These results together indicate that clofibrate may prolong or enhance the hormonal action of endogenous E_2. Along these lines, attention is drawn towards the alterations of VTG mRNA abundance in hepatocytes from a male as well as a female fish found in this study. The examined female trout was at the beginning of the vitellogenesis because VTG mRNA could be detected in all treatments including the solvent control at the first time point. Over the course of the experiment, the VTG mRNA abundance decreased over time in all treatments. This indicates either a degradation of the mRNA and an apparent lack of VTG mRNA production due to the loss of the endogenous E_2 supply, or an esterification of E_2 due to CA as described by Xu et al (2001). However, VTG mRNA levels dropped to the same extent in both CA-treated cells as well as in the solvent control. This observation points toward a CA-independent mechanisms of VTG mRNA decline although with the PCR methods applied, the cause of the decline in VTG mRNA cannot be deduced. A CA dependent induction of VTG mRNA was, however, detected upon 78 h of exposure to CA. Whether this induction was due to back-conversion of esterified

E_2 or due to some other way of VTG mRNA induction by CA needs further exploration. In the hepatocytes used in this report, further investigations in this regards were hampered by the fact that the results of hepatocytes of the vitellogenic fish could not be replicated in hepatocytes from a non-vitellogenic fish with or without the intentional addition of E_2. The observed difficulty in replicating this response is similar to the results of a microarray study where in only two of six human hepatocyte cultures a significant up-regulation of CYP4A was observed (Richert *et al*, 2003).

In hepatocytes from the male trout, no VTG mRNA was observed within the first 30 h of exposure. After 54 h in the non-treated cells as well as in the solvent control, low VTG levels were detected. Evaporation through the air filled space from the exposed wells to the solvent control is very unlikely, for CA has a very small Henry's law constant ($2{,}19 \times 10^{-8}$ atm-m^3/mol at 25°C). Despite the elevated VTG mRNA expression levels in the solvent controls after ≥54 h, a specific response to CA was found. The induction of VTG mRNA was significant from 10^{-7} M to 10^{-5} M CA. At the lowest tested CA concentration (10^{-8} M) no VTG mRNA was detected in contrast to the results in the cells from the female. The mechanism underlying the VTG response to CA in male fish is not yet understood, apparently an indirect activation of VTG transcription occurred. Future investigations focussing on the mechanisms are necessary and could derive benefit from the hepatocyte model.

Taken together, CA caused no acute cytotoxic effect on primary hepatocytes from rainbow trout as well as no influence on basal functions of liver cells, namely the expression, at least on the mRNA level, of serum albumin, transferrin and CYP3A27. Furthermore, no evidence for an increased lipoprotein lipase expression was found. Thus, the typical effect of CA as hypolipidemic drug was not detected. However, an indication for a slight mitochondria proliferative effect was found. A CA dependent VTG response in both sexes was detected for the first time.

Consequences for Chapter 3.4

Together with Chapters 3.1 and 3.2 of this thesis, this chapter further characterized the primary rainbow trout hepatocytes as a model system to study gene expression. Specifically, it showed that presumed effects, such as the induction of lipoprotein lipase (LPL) due to clofibric acid (CA), may not be confirmed while other complex mechanisms, such as a CA-dependent induction of vitellogenin (VTG) mRNA, may occur. Thus, this chapter illustrates the potential usefulness of a cDNA-array in simultaneously analysing larger numbers of genes. As in Chapter 3.2, this chapter further established an exposure time of about 54 h as appropriate for obtaining maximal mRNA levels for the genes selected thus far. Taken together, the basics were given for the preparation of a user-defined rainbow trout cDNA-array and its use in comprehensive explorations on gene expression in rainbow trout hepatocytes.

Chapter 3.4

Development and application of a user-defined cDNA-array

Introduction

The recent emergence of toxicogenomics, i.e. the search for understanding the consequences of altered gene expression upon exposure of cells and tissues to toxic chemicals, gives rise to technological advances that are beginning to revolutionize environmental toxicology. One such advance is the development of the cDNA microarray technology. A cDNA-array is based on immobilized DNA fragments on a nylon membrane or glass slide and hybridization with labelled cDNA synthesized from total or messenger RNA of a cell or tissue sample. It allows to detect the alteration of the expression of thousands of genes simultaneously. Previous studies with mouse (Bartosiewicz *et al*, 2001) and human genes (Pennie *et al*, 2001) have illustrated the potential of cDNA arrays to effectively elucidate the complex mode by which genes are regulated by environmental toxicants. Recently, a few studies were made on ecotoxicological effects in several fish species (Larkin *et al*, 2003b; Williams *et al*, 2003; Koskinen *et al*, 2004).

Whole genome arrays with thousands of genes produce a lot of information particularly useful for identifying regulated genes or groups of genes out of a large pool. For detailed investigations, such organ- or tissue specific regulations, however, small targeted gene arrays appear valuable. In contrast to whole genome arrays, these arrays carry genes that have been specifically selected for exploring the question at hand. For example, a small user-defined array could be useful for investigating a special, contoured biological effect such as an estrogenic response. It thus was the goal of this chapter of the thesis to develop a user-defined rainbow trout cDNA-array with genes particularly important in liver function, such as metabolism and an estrogenic response.

Results

The first step in the establishment of the rainbow trout liver cDNA-array was the selection of genes considered to be important. The selection was based on available literature and on the results obtained in Chapters 3.2 and 3.3. The genes selected were chosen so as to cover e.g. estrogenic responsive genes, genes involved in xenobiotic metabolism and genes crucial in normal liver function (see appendix, table A1, A2). The next step was the derivation of the DNA fragments to be spotted on glass slides to obtain the arrays. The procedures for this are

described in section 2.9.2 in the Materials and Methods Chapter. Based on these fragments, two sets of arrays were printed in order to establish the rainbow trout liver arrays. In the following, the design of each of these two sets of arrays is described along with the steps taken to optimize the outcome and evaluation of the arrays and their respective gene expression results.

First set of arrays

Array Design

The first spotting resulted in a set of slides with 14 cDNA fragments of interest and 2 hybridization control cDNAs (table A1 of genes in the appendix). The genes were selected based on their known or suspected role in response to estrogens or in xenobiotic metabolism. As well, genes were selected due to their presumed stable constitutive expression (for use as house-keeping genes). The two hybridization controls were a poly (d)A oligonucleotide and a lambda fragment from a *Hin*dIII digest. The linear amplified, labelled DNA from the lambda fragment was added to the labelled samples before hybridization to examine the quality of the hybridization.

Each DNA fragment was spotted in four replicates and all probes were spotted a second time close to the first four replicates of the same slide (eight replicates of every gene in total). The glass slides had been pre-treated with poly-L-lysine as described in Chapter 2.9.3. The spotting concentration of DNA was 400 ng/µL for all spots. Upon spotting, the spot quality was determined using SYBRgreen II which has a specific affinity to ssDNA and which can be detected by its fluorescence (Figure 3.4.1).

Hybridization

First hybridizations were done using an Array Hybridization Buffer purchased by Sigma. This buffer had a conspicuous high viscosity and the cover slips could only be removed with the aid of tweezers after hybridization. Thus, the hybridization buffer (Hyb-buffer) was changed to the self-prepared Hyb-buffer (see Chapter 2.9.8). All further experiments were performed with the self-prepared Hyb-buffer.

After hybridization, quality criteria have to be set so as to decide which spots should be included into the analysis (Winzeler *et al*, 1999). The most common criterion used is a fluorescent signal/noise (background) ratio ≥ 2. This means that the fluorescent intensity of the signal is at least 100 % greater than the fluorescent intensity in the neighbourhood of the spot (background).

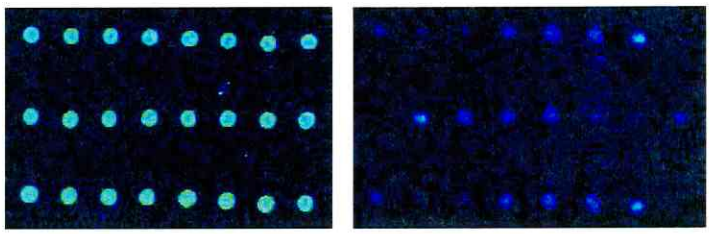

Figure 3.4.1 Fluorescent images of two subarrays (separate parts of the whole array, defined by printing with the same pin) from a SYBRgreen II stained slide. The left picture represents a good staining of gene spots. For comparison, the right picture shows a lack of staining of spots that were treated with spotting buffer (and no DNA fragments) alone. Here, only minor non-specific staining occurred. Upon visualisation, SYBRgreen II staining was washed off and hybridization performed as described in Chapter 2.9.8.

Gene expression determined by the first set of arrays

The first set of slides was used to investigate effects elicited by 10^{-7} M 17β-estradiol (E_2) and 10^{-7} M 17α-ethinylestradiol (EE_2) upon exposure for 54 h in primary rainbow trout hepatocytes. The 54 h of exposure were chosen because previous experiments (see Chapters 3.2. and 3.3) had shown that gene expression was generally at its maximum after 54 h of exposure or more. One dye swap experiment for each substance was performed with samples obtained from one hepatocyte isolation. An additional array hybridization without a dye swap was performed with samples from another hepatocyte isolation and exposure to EE_2. Dye swap experiments are useful for removing systematic dye bias, which can result from different dye incorporation efficiency and the different quantum yield of the dyes (Kerr & Churchill, 2001; Dobbin *et al*, 2003; Sanchez-Cabo *et al*, 2004). In a dye swap experiment, treated and control RNA are first labelled with two different dyes (such as Cyanine 3, Cy3 and Cyanine 5, Cy5) in a given combination. Then, in a second experiment, the treated and the control RNA are each labelled with the other dyes so as to have each of the RNAs labelled with one of the dyes once.

Different routes are possible in order to normalize array gene expression data. One is the use of house-keeping genes. The other is the mathematical algorithm LOWESS (Robust locally weighted regression and smoothing scatterplots, Cleveland, 1979). In brief, LOWESS presumes that the majority of all spots are not regulated and therefore should have the same value. The real values are weighted and fitted. This computation is referred to as locally weighted regression and leads to a smoothed scatterplot. Data evaluated either with house-keeping genes or the LOWESS algorithm led to the same set of up-regulated genes in this first set of arrays (Table 3.4.1), with the genes β-actin and cytochrome-c-oxidase subunit VIa being used as house-keeping genes. No down-regulated genes were found.

Table 3.4.1 Overview of the performed experiments and the results using the first set of arrays

Experiment description	**Exposure**	**up-regulated genes**
1 dye swap with a single fish	10^{-7}M E_2	HSP 70 Glucose-6-Phosphatase
1 dye swap with a single fish 1 hybridization from another fish	10^{-7}M EE_2	Estrogen receptor HSP 70 Cyclooxygenase 2 VEP β Retinol binding protein

Exposure to 10^{-7} M E_2 for 54 h led to a 3-fold increase of glucose-6-phosphatase mRNA abundance in both hybridizations of the dye swap experiment as well as a 4-fold induction of HSP 70 mRNA abundance in one hybridization and a 63-fold induction in the other hybridization of the dye swap experiment. Also in hepatocytes exposed to 10^{-7} M EE_2 for 54 h, an increase of HSP 70 mRNA abundance was found in both hybridizations of the dye swap, but only approximately 2.5-fold. Beyond HSP 70 and glucose-6-phosphatase, four other genes were identified to be up-regulated, namely cyclooxygenase 2, VEPβ, retinol binding protein and the estrogen receptor (Figure 3.4.2). In this study the latter of them was identified as not distinctly regulated as it just reached, but not exceeded, the ratio = 2 of the normalized fluorescent signal from sample and control. However, it is unlikely to be a false-positive, because of its high reproducibility determined by the very small variance between hybridizations (5%) and its known function in the regulation of an estrogenic response.

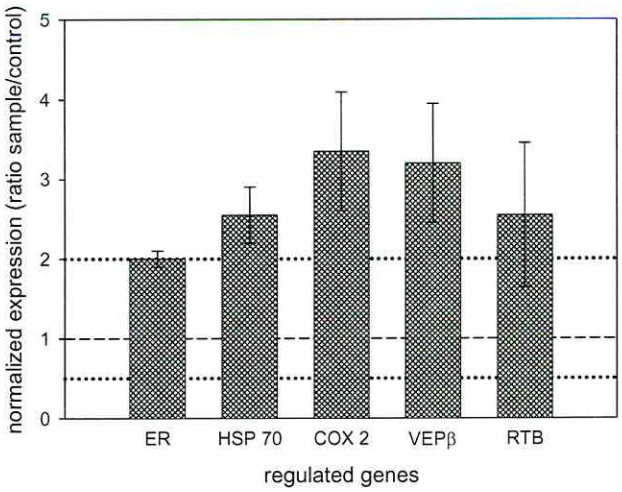

Figure 3.4.2 Regulation of mRNA abundance of five genes upon exposure of hepatocytes from two independent experiments (N=2) for 54 h to 10^{-7} M EE_2, analyzed with a dye swap and an independent hybridization. Each bar represents the mean ratio of the normalized fluorescent signal sample/solvent control ± deviation from the mean. Dashed line represents the ratio 1, meaning no regulation. The dotted lines represent the ratios 0.5 and 2, respectively and comprise the range beyond which regulation is postulated to be significant.

ER = estrogen receptor

HSP 70 = heat shock protein 70

COX 2 = cyclooxygenase 2

VEPβ = vitelline envelope protein β

RTB = retinol binding protein

Second set of arrays

Array Design

The second spotting resulted in a set of slides with 48 cDNA fragments of interest and the DNA contamination controls actin genomic band and growth hormone 1 intron C (table A2 of genes in the appendix). As well, poly d(A) oligo was again included as a control of hybridization quality. Commercial controls (Lucidea Universal ScoreCard from Amersham Biosciences, UK) were also included. This Amersham control kit can serve as an external calibration curve on the slide. It was tested for its usefulness in the user-defined rainbow trout cDNA-array. Further, negative spot controls were included, namely water (i.e. 1 x spot buffer) and undiluted 2 x spot buffer. The number of negative spot controls corresponded to 15.6 % of the total number of spots. In addition, cDNA fragments from VEPα, estrogen receptor and the oligo poly d(A) were spotted in three different concentrations (400 ng/µl, 200 ng/µl, 100 ng/µl) to investigate the concentration needed for detecting differential expression on the arrays. Each gene was spotted in three replicates and all probes were spotted a second time on the bottom of the same slide (six replicates of every gene in total).

Gene expression determined by the second set of arrays

The second set of slides was used for investigation of effects elicited by 10^{-7} M 17β-estradiol (E_2) for 54 h in exposed hepatocytes. One dye swap experiment was evaluated. A section of a fluorescence image is shown in Figure 3.4.3.

Figure 3.4.3 Exemplary fluorescent image from two subarrays from a hybridized slide.

The raw data were evaluated using LOWESS normalization. The LOWESS algorithm for normalization includes all spots (inclusive the replicates). Normalization of the Cy intensities is necessary because of occurring dye bias due to, amongst other factors, slightly different RNA amounts, cDNA synthesis efficiency, dye incorporation efficiency and the different quantum yield of the dyes. The data cloud is transformed on the function y = x by using the LOWESS normalization. This function represents the same fluorescence intensity of both dyes. All spots lying on or close-by the function y = x are defined as non-regulated. The area just above and below this line is defined by the functions y = 2x and y = ½x. This means that all spots outside of this area show significantly different intensities of the two dyes and are therefore defined as regulated. The need for normalization is demonstrated in Figure 3.4.4. RNA from the same sample was divided and one part was labelled with Cy3 and the other part with Cy5. The hybridization of both labelled cDNAs on one slide yielded information about the systematically occurring bias. This bias was indicated by the permanently greater fluorescence signal for Cy5 than for Cy3 resulting in a shift of the data cloud above the function y = x (the diagonal line in Figure 3.4.4). The transformation step LOWESS for normalizing the data abolished this shift (Figure 3.4.4). The normalization step allows the levelling of all non-biological disturbances in the ratio of the two fluorescent dyes. Hence, an evaluation of the data regarding gene regulation caused solely by the exposure to the test compound is now possible.

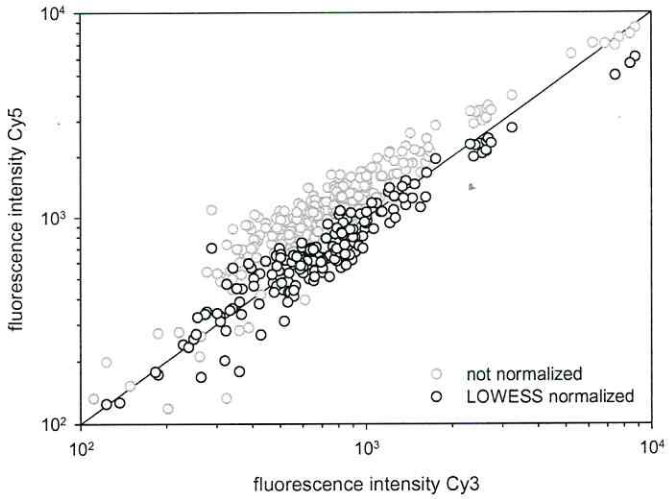

Figure 3.4.4 Comparison of the fluorescence intensity of the Cy dyes of non-normalized raw data and LOWESS normalized data. Untreated total RNA was divided and labelled with Cy3 and Cy5, respectively. Differences of fluorescence intensities between both dyes could be caused only by different incorporation efficiencies during the labelling and different quantum yields of both dyes. The diagonal line represents the function y = x.

An example of LOWESS-normalized array data is shown in Figure 3.4.5, which is based on samples obtained from rainbow trout hepatocytes exposed for 54 h to 0.5% EtOH as control and 10^{-7} M E_2 as treatment. In contrast to Figure 3.4.4, the replicate spots were averaged. The majority of the investigated genes were non-regulated and only four genes were found to be up-regulated, namely CYP11B, VEPα, VEPβ and VEPγ. CYP11B mRNA abundance was 3-fold higher in the treated sample than in the control. This member of the cytochrome P450 superfamily, also called 11β-hydroxylase, is involved in the steroidogenic pathway of stress hormones (Okamoto & Nonaka, 1990). It catalyses the hydroxylation of 11-deoxycortisol to cortisol. The detected up-regulation of the three VEPs α, β, γ showed the same pattern as observed by qPCR in Chapter 3.2. VEPγ mRNA abundance was 4.7-fold induced compared to the control, whereas VEPβ mRNA level was 3.8-fold higher and VEPα mRNA abundance was 3-fold higher than the solvent control. This induction pattern is generally in accordance to the results obtained by qPCR (Chapter 3.2, Figure 3.2.3). Interestingly, VTG showed no significant response upon exposure to 10^{-7} M E_2 compared to the solvent control.

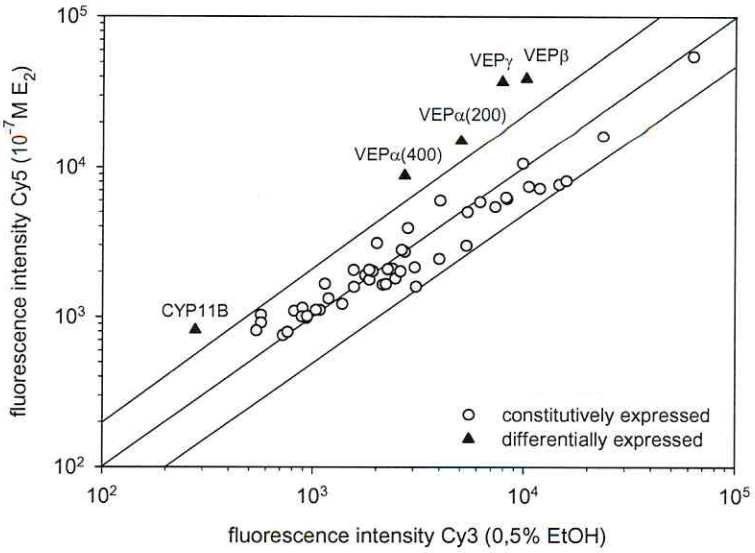

Figure 3.4.5 Fluorescent signals after hybridization with combined samples treated with 10^{-7} M E_2 and solvent control (0.5% EtOH) for 54 h. Data are normalized with LOWESS algorithm and by averaging the replicate spots. Grey dots represent constitutively expressed genes and triangles represent genes that showed a differential regulation. Numbers behind VEPα indicate the spotting concentration of this gene.

The DNA fragment from VEPα was spotted in three different concentrations (400 ng/µl, 200 ng/µl, 100 ng/µl). Except for the lowest concentration, a signal/noise ratio > 2 was derived, thus the spots including 400 ng/µl and 200 ng/µl were analysed and showed an up-regulation upon exposure to 10^{-7} M E_2 of approximately 3-fold. No significant difference between the derived induction values was found. The lowest concentration 100 ng/µl yielded only spots of minor quality and was not included into analysis.

The commercial control kit Scorecard consists of a set of artificial genes. Their mRNA proved to not cross-hybridize with RNA from human, mouse, rat, yeast, Arabidopsis thaliana, palm and several bacteria. However, it was found to not be useful in a rainbow trout array, because cross-hybridization of trout mRNA to the spots from the control kit occurred (Figure 3.4.6).

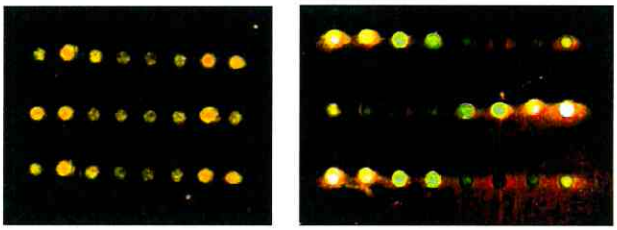

Figure 3.4.6 Fluorescent images of the same subarray containing the spots for the calibration curve from two independent slides. The left picture represents cross-hybridization of rainbow trout cDNA without having spiked it with the commercial mRNA. Red dots indicate a moderate intensity. For comparison, the right picture shows the specific hybridization with the spiked mRNA.

Discussion

The application of nucleotide arrays in ecotoxicology is a powerful technique for identifying patterns of regulated genes upon pollutant exposure. In this study, user-defined rainbow trout cDNA-arrays were developed and their usefulness was investigated for studying gene expression in rainbow trout liver upon exposure to estrogens. Two sets of arrays were spotted and validated. Glass slides, coated with poly-L-lysine, were used for spotting of cDNA fragments. Several replicates of the fragments were spotted (eight in the first set and six in the second set), since by repeated positioning of the same fragment on the array, the accuracy of the measured fluorescence signals can be increased by averaging them. Repeated spotting also can help minimize difficulties caused by flaws of, or lint on, the slide surface. The experiments carried out with these produced arrays were done using the two color technique. Thus, control and sample RNAs were labelled with different fluorescent dyes, which were measured with two channels in the Perkin-Elmer ScanArray Express scanner, respectively.

After generation of scanning data, normalization is an important next step. Several normalization methods were reviewed in Quackenbush (2002) and Hackl *et al.* (2004). Data evaluation needs to start with the normalization as it removes all variations that can be introduced into the procedure but do not relate to a biological effect (Quackenbush, 2002; Yang *et al*, 2002). For example, a difference between the intensities of the two Cy dyes for a particular gene may be due to differences in expression levels between the samples or differences in dye incorporation efficiency or different quantum yield between the dyes (Gruber *et al*, 2000; Dobbin *et al*, 2003). Other factors introducing variability are the used RNA amount and varying cDNA efficiencies resulting in different cDNA amounts applied on the array. These might mask the biologically caused difference in gene expression. Small nucleotid arrays (as developed in this study) can be normalized using house-keeping genes (Winzeler *et al*, 1999). These house-keeping genes are supposed to not be regulated under the experimental conditions. β-actin and cytochrome-c-oxidase subunit VIa were considered as house-keeping genes for the first set of slides. β-actin was validated using qPCR and found to not be regulated in rainbow trout hepatocytes upon E_2, EE_2 and CA exposure over time and concentration (see Chapter 3.2 and 3.3). Cytochrome-c-oxidase subunit VIa was not validated in previous studies on primary rainbow trout hepatocytes. In the present study, cytochrome-c-oxidase subunit VIa showed only little variation upon E_2 and EE_2. However, the literature regarding the cytochrome-c-oxidase family is controversial. Another subunit, cytochrome-c-oxidase subunit Ia was used as normalization base in qPCR performed from rat tissues upon phytoestrogenic exposure (Diel *et al*, 2004). On the other hand Chen *et al.* (2003) found an up-regulation of the mRNA abundance of the cytochrome-c-oxidase subunits I, II and III in EE_2 exposed rat hepatocytes. Nothing has been known so far about the behavior and regulation of the cytochrome-c-oxidases upon estrogen exposure in rainbow trout. Thus, every considered house-keeping gene should be validated under the specified experimental conditions. Ideally, this should be done using

qPCR because qPCR is more accurate than conventional PCR (Williams *et al*, 2003). Furthermore, two house-keeping genes may not be sufficient to obtain reliable results (Winzeler *et al*, 1999). The suggestion evolving from these results is to include in such a small array more than two house-keeping genes with as many of them as possible being validated by qPCR.

Another possibility to normalizing microarray data is the mathematical LOWESS algorithm which represents a robust locally weighted linear regression analysis (Cleveland, 1979). This normalization method is widespread in use for correcting the dyes bias of microarray data (e.g. Yang *et al*, 2002). The raw data from the first set of slides were evaluated with the two mentioned house-keeping genes and in parallel with the LOWESS normalization. With both normalization methods, the same set of regulated genes was found, with all of them being up-regulated (see table 3.4.1). The experiments with slides from the first set (14 genes) contained dye swaps with samples from E_2 and EE_2 exposed trout hepatocytes, respectively. The exposure of 10^{-7} M E_2 for 54 h led to the up-regulation of the enzyme glucose-6-phosphatase and the heat-shock-protein HSP 70. Glucose-6-phosphatase is one key enzyme in the gluconeogenesis and described as estrogen regulated in rats (Moorthy *et al*, 2004) and fish (freshwater murrel, Sehgal & Goswami, 2001) *in vivo*. HSP 70 was also found to be up-regulated upon exposure to 10^{-7} M EE_2. The protein being coded for by this gene is involved in stress response (Iwama *et al*, 1999) and has not yet been described as estrogen regulated in rainboe trout hepatocytes. Cyclooxygenase 2, which is responsible for the prostaglandin synthesis, was observed to be up-regulated by EE_2 for the first time. Currently, Tamura *et al*. (2004) discovered the estrogen receptor mediated up-regulation of cyclooxygenase 2 by E_2 in primary human uterine microvascular endothelial cells. Furthermore, retinol binding protein (transport of vitamin A) was found to be up-regulated upon 10^{-7} M EE_2 exposure which is in contrast to the observed downregulation by E_2 in Gilthead Bream *in vivo* (Funkenstein *et al*, 2000). Yet, a different influence on gene expression by EE_2 and E_2 has also been observed for serum albumin: only EE_2 caused a response in serum albumin mRNA abundance in primary hepatocytes from rainbow trout but E_2 did not (see Chapter 3.2). Apparently, E_2 and EE_2 do not necessarily cause the same effects in rainbow trout hepatocytes. The two genes, estrogen receptor and VEPβ, that were up-regulated upon 10^{-7} M EE_2 are known to be under estrogenic control (MacKay *et al*, 1996; Westerlund *et al*, 2001, Chapter 3.2). Taken together, a few of the genes found to be up-regulated, namely estrogen receptor, VEPβ, cyclooxygenase 2 were described as directly regulated by estrogens in the literature and others to be indirectly regulated such as retinol binding protein and glucose-6-phosphatase. HSP 70, which was found to be up-regulated by E_2 and EE_2 in this study, has not yet been described to be under estrogenic control in rainbow trout. However, further studies with qPCR in terms of reproducibility with another measuring method are required for validation. In addition, the use of ICI 182,780 could give information as to whether the response of HSP 70 and cyclooxygenase 2 in rainbow trout hepatocytes is estrogen receptor mediated.

According to the evaluation of the first set of arrays, data from the second set of arrays (containing 48 genes) were also normalized with the LOWESS algorithm. The abolishment of the occurring systematic dye bias (Figure 3.4.4) due to the normalization procedure LOWESS was shown to be eligible for the present data set. The time- and cost-consuming validation of a substantial amount of house-keeping genes is not necessary when using the LOWESS transformation. Hence, the data can be further validated regarding biological effects without the need for tedious transformations.

The experiments with the second set of arrays contained only samples from E_2 exposed hepatocytes. The up-regulated gene CYP11B has not yet been described as estrogen regulated in fish. Further research is necessary for validating this finding. The detected estrogen-dependent up-regulation of the three VEPs α, β, γ in this study was validated by qPCR (Chapter 3.2) and generally displayed the same pattern of induction. This confirms the comparability of the two sensitive molecular techniques, qPCR and nucleotide array. However, fragments for transferrin and albumin have also been spotted on the array, but no response upon E_2 (as detected with qPCR in Chapter 3.2) was found. This could be due to the methodological limitations in identifying down-regulated genes: down-regulation in the fluorescent signals is smaller compared to a multiplication of the signal during an up-regulation. It is for this reason that the qPCR is more sensitive than the nucleotide array technology at least in terms of detecting and monitoring down-regulated genes. It has to be mentioned, that VTG was surprisingly not found to be regulated although it was validated as robust estrogenic biomarker by means of qPCR in Chapter 3.2. Despite that all fragments cloned into E.coli were sequenced, the clone for VTG may not be intact. Another verification by sequencing should be performed before the next spotting event.

Beyond the investigation of two normalization methods and the identification of regulated genes, the impact of various DNA spotting concentrations was evaluated. The investigation of three DNA spotting concentrations revealed that the used concentration of 400 ng/µL for all other DNA fragments can be replaced with half the concentration 200 ng/µL. This is in accordance to Bartosiewicz *et al.* (2000), who used 200 ng/µL for all spotted PCR fragments. Larkin *et al.* (2003b) used only 160 ng/µL cDNA template for spotting. The lowest concentration included in the present work, 100 ng/µL, was not able to deliver a good fluorescent signal. This might be due to insufficient binding sites for labelled cDNA to generate a fluorescence signal above background level. For this reason, 200 ng/µl as spotting concentration should be taken into account in future spotting events.

Taken together, a user-defined cDNA-array was developed and validated. The two normalization methods using LOWESS and house-keeping genes, respectively, were shown to lead to the same results but the LOWESS method is more versatile and easy to apply. Furthermore, well-known estrogenic marker genes, as well as two genes not yet known to be regulated by estrogens in fish were identified with the array developed in this study. Based on

the presented results, it is recommended to include more than two house-keeping genes for an optimized user-defined array (at least 40% of the whole number of spots) for appropriate data normalization. A user-defined array allows to focus on the genes of interest in contrast to generating data from thousands of genes with commercially available arrays. It represents an efficient method for determining fingerprints of gene expression elicited by ecotoxicants in particular tissues or pathways.

Chapter 4

Concluding remarks and future directions

Ecotoxicants can elicit their effects by changes to the level of gene expression, either by direct interaction with transcriptional regulation or as part of a cellular response to chemical stress. Thus, analysis of the expression of responsive genes after exposure to ecotoxicants allows to identify potential hazardous effects and regulatory mechanisms. In light of this, this thesis aimed at establishing rainbow trout hepatocytes for studying multiple gene expression and developing a user-defined cDNA-array for rapid detection of estrogenic effects on the gene expression level. Four major steps were pursued to accomplish this goal (Figure 4.1). At first, the test system, primary rainbow trout hepatocyte culture, was characterized with regard to cell viability and metabolic activity throughout the time of culturing. A versatile, non-invasive fluorescent assay, using alamarBlue and CFDA-AM as indicator dyes was established for this purpose (step 2). Afterwards, gene responses upon exposure to the natural hormone 17β-estradiol, to the synthetic hormone 17α-ethinylestradiol and the pharmaceutical clofibric acid were investigated by means of qPCR for validating a small set of genes with regard to liver function and estrogenic reponse. This small set of genes and many more identified by literature search were finally used to design the user-defined cDNA-array.

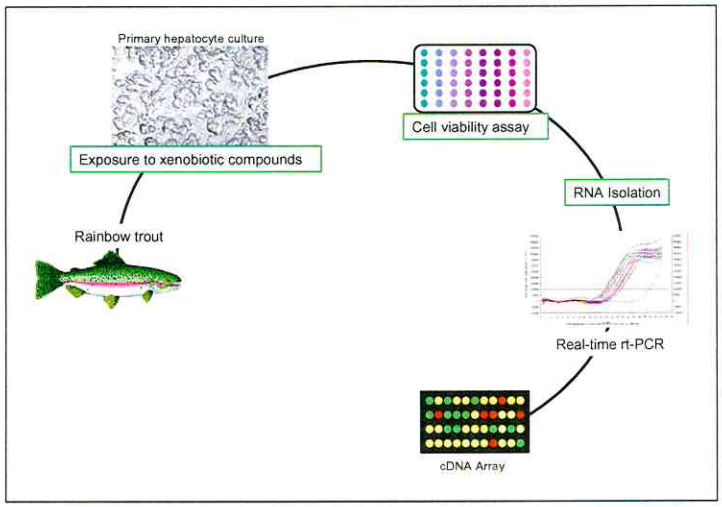

Figure 4.1 Experimental steps used in this thesis for investigating estrogenic effects on gene expression level in primary rainbow trout hepatocyte cultures.

The cell viability assay validated in the first part of this thesis allows to monitor potential hazardous impacts of individual ecotoxicants or complex environmental samples on cell vitality. The non-invasive assay, using the two fluorescent dyes alamarBlue and CFDA-AM, was for the first time applied to primary hepatocyte cultures from rainbow trout. By means of this assay, the culture medium L-15 was identified to more stably support hepatocyte viability than the more complex culture medium M199. As well, the assay proved valuable for detecting acutely toxic effects, such as of the estrogen receptor antagonist ICI 182,780. It can be used repeatedly on the same set of cells and was shown to not impact on gene expression. Inasmuch as an impaired cell vitality may affect gene expression, the cell viability assay should be generally applied in the future prior to RNA extraction and analysis of gene expression. One problem arising in the application to primary hepatocyte cultures was a somewhat lower sensitivity of CFDA-AM if compared to the same assay applied to a cultured fish cell line. The difference in sensitivity was hypothesized to be, at least in part, due to the methodology: in the primary cells, a half-medium change instead of a full medium change was performed to keep the handling stress on the rather loosely attached hepatocytes to a minimum. Thus esterases leaking out of cells and potentially metabolizing CFDA-AM to its fluorescent product, carboxy fluorescein (CF), were only partially removed prior to addition of the dyes. With careful handling, it might be possible in the future to remove more than half the medium in order to diminish this limiting effect. Despite this drawback of the current method, application of the two dyes in combination is useful because it helps decipher specific effects. One example of this was seen in Chapter 3.3 where an impact of clofibric acid on mitochondria was identified based on the two-dye assay approach.

Three ecotoxicants, namely 17β-estradiol (E_2), 17α-ethinylestradiol (EE_2) and clofibric acid (CA) as well as appropriate solvent controls were investigated for their impact on cell viability and the expression of six (CA) to eight (E_2 and EE_2) genes in primary rainbow trout hepatocytes in a dose- and time-dependent manner. Among the genes monitored and with regard to mRNA levels in the solvent controls or blanks, a significant decline in serum albumin mRNA abundance over the course of the experiment and a drop in CYP3A27 mRNA level between 24 and 48 h of culture was observed. A loss of CYP3A27 mRNA following a collagenase perfusion procedure, as it was done in this work, was previously reported for rat hepatocytes (Bissell, 1976). In contrast, the decrease in serum albumin mRNA level could be due to bacterial lipopolysaccharide in the collagenase used during the hepatocyte isolation. Lipopolysaccharide has recently been shown to stop the expression of albumin mRNA in rats via a not fully understood pathway (Paine & Andreakos, 2004). In further experiments, a lipopolysaccharide free collagenase (e.g. Liberase from Roche) should therefore be evaluated.

Genes specifically explored upon E_2 and EE_2 exposure were β-actin, vitellogenin, vitelline envelope protein α, β, γ, CYP3A27, transferrin and serum albumin. Firstly, the co-exposure of E_2 or EE_2 treated cells with the synthetic estrogen receptor antagonist ICI 182,780 led to a decrease in cell viability at high antagonist concentrations and confirmed the usefulness of the

cell viability assay discussed above. Secondly, the expression of β-actin was identified as not affected by either E_2 or EE_2 and thus was confirmed as constitutively expressed under the experimental conditions used in this thesis. Hence, β-actin can be used as house-keeping gene in gene expression analysis upon estrogen exposure of primary hepatocytes from rainbow trout in future experiments. Thirdly, VTG mRNA abundance was found to be a more robust biomarker for estrogenic responses than the three VEPs α, β, γ. However, the VEPs α, β, γ appeared to be expressed more sensitively with regard to time. It thus would be interesting to investigate the different ways of regulation of the VEPs in more detail in the future. Fourthly, although the decline in culture of mRNA levels of serum albumin and CYP3A27 made observing effects on gene expression by E_2 and EE_2 difficult, a significant increase in CYP3A27 mRNA abundance after co-exposure to ICI 182,780 and E_2 and EE_2 was identified. Hence, the involvement of the estrogen receptor in the regulation of CYP3A27 was shown in this thesis for the first time. Future studies should extent these findings to the level of the protein and possibly enzyme function.

The pharmaceutical clofibric acid (CA) is both of environmental concern and so far not adequately investigated in fish. Interestingly, no induction of lipoprotein lipase (LPL) was found upon exposure to CA although LPL is one key enzyme in fatty acid catabolism and one target of the fibrates to which CA belongs. Future studies should substitute LPL by genes coding e.g. for an enzyme of the cytosolic fatty acid metabolism, such as acyl CoA thioesterase, since this enzyme was found to be up-regulated in hepatocytes from human, rat and mouse (Richert *et al*, 2003). The alterations in VTG mRNA abundance found in this study were difficult to consistently repeat and thus far cannot be sufficient explained. For this reason, future experiments are necessary and should at first focus on the presence of PPARα mRNA in rainbow trout hepatocytes in culture. In addition, based on the results of this thesis, the use of female trout that are already in the vitellogenic phase and therefore have elevated levels of endogenous E_2 and VTG in the liver would be of advantage. The use of an estrogen receptor antagonist as ICI 182,780 can reveal a potential participation of the estrogen receptor. As well, adding more males to this study would help raise the understanding of the underlying mechanism.

The final step in this thesis work contained the design, production and application of a user-defined cDNA-array specific for rainbow trout liver. The importance of normalizing the raw data obtained from a cDNA-array was shown as well as the comparability of the two normalization methods, using house-keeping genes und LOWESS. The method via house-keeping genes includes the risk of dependency on only a limited number of signals thereby not all genes selected as house-keeping genes might be appropriate for all experimental conditions. For this reason, genes used for this method need to be carefully checked for their constitutive expression under all the experimental conditions relevant. In future array designs, numerous house-keeping genes could be included, however, it will be much cheaper and faster to use LOWESS for normalization. In this study, the LOWESS normalization procedure was found to

work well with a little as 160 signals. Thus, LOWESS can also be used for small, targeted arrays. With the arrays, two genes were found for the first time to be up-regulated by E_2 (HSP 70, CYP11B) in fish. As well, two genes were found for the first time to be up-regulated by EE_2 (HSP 70, Cyclooxygenase 2). These findings confirm the usefulness of a tissue-specific array, since it is a rapid method to derive new insights. However, further experiments should be performed using qPCR for the identified genes to specifically confirm these results. In addition to identifying relevant genes, the developed cDNA-array can be used to generate fingerprints from well-known estrogenic substances, e.g. E_2, EE_2, nonylphenol, DDT as started in this work and subsequently used to screen complex environmental samples for estrogenic responses by comparing the fingerprints with the obtained expression pattern.

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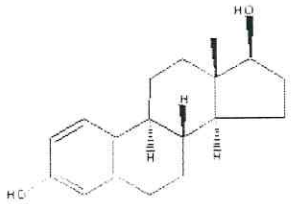
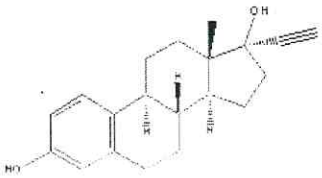
Appendix

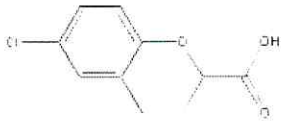
Chemical structure of the three model substances investigated in this thesis.



A 17β-estradiol



B 17α-ethinylestradiol



C clofibric acid

Figure A1 Chemical structure of **A** 17β-estradiol, **B** 17α-ethinylestradiol, **C** clofibric acid (chemical name: 2-(p-Chlorophenoxy)-2-methylpropionic acid)

Table A1 Spotted cDNA Fragments on the first set of slides (Chapter 3.4)

Gene Name	GenBank Accession	Fragment Size	Protein Function
HSP 70	AB062281	530	Heat shock protein
Metallothionein	M18104	371	Homeostasis of the essentiell metal concentrations
Vitellogenin	M27651	488	Precursor for the egg yolk protein
Estrogen Receptor α	AJ289883	502	Nuclear receptor for estrogens
VEP β	AF231707	537	Component of the oocyte envelope
Aryl-Hydrocarbon-Receptor	AF499010	522	Mediation of CYP1A induction
Glucocorticoid Receptor	Z54210	545	Cytosolic receptor for glucocorticoids
Cyp 2M1	U16657	454	Involved in fatty acid hydrolysis
Cyclooxygenase 2	AJ238307	502	Prostaglandin synthesis
Retinol binding protein	AF257326	477	Transport protein for vitamine A
Glucose-6-Phosphatase	AF120150	532	Enzyme in the gluconeogenese
Transferrin	D89083	525	Transport protein for iron
Cytochrome-C-Oxydase Subunit VIA	U83980	469	Respiratory chain (house-keeping gene)
β-Actin	AF157514	540	(house-keeping gene)
Actin genomic band	primer sequence provided by T. Mommsen, University of Vancouver, Canada	540	DNA contanimation control
Lambda DNA		564	Hybridization control
Poly A		50	Hybridization control

Primer sequences are provided in table A2

Table A2 Spotted cDNA Fragments on the second set of slides (Chapter 3.4)

Gene Name Array 2004	Genbank Accession	Fragment Size	Protein Function	Primer Sequences (5' – 3')
HSP 70	AB062281	530	Heat shock protein	CTT ACC TGG GCC AGA AGG T TGA AGA GGT CGG AAC ACA T
Metallothionein	M18104	371	Homeostasis of the essentiell metal concentrations	CAA CAC ACC ACT GAC ACC C AAG TTA CAT GCA ATT TAT T
Vitellogenin	M27651	488	Precursor for the egg yolk protein	ATG AGA GCA GTA GTA CTT G TCT TGC ACA CTC CCT GAG C
Estrogen Receptor α	AJ289883	502	intracellular receptor for estrogens	GAT CAT GTG CAC CAG CTC CT GTA CCA CTA CGG AGT TTG GTC CT
VEP β	AF231707	537	Component of the oocyte envelope	CTT CTG TCT TTG CCA TCT AC TGG TCA GGT AAC GGT CAT
Aryl-Hydrocarbon-Receptor	AF499010	522	cytosolic multiprotein complex, ligand-activateed transcription factor	CAG CGA AGG GAG CGG TAA TGG ACC CGG CCA GTG ATA
Glucocorticoid Receptor	Z54210	545	Cytosolic receptor for glucocorticoids	CCT TGG CGA CAA TAC AAT AGC TCG ACA TCC CTG ATC
CYP2M1	U16657	454	Involved in fatty acid hydrolysis	TGT CCT CAG CAG CCC TAT GTA TCG CTG GAC TTC GTG
Cyclooxygenase 2	AJ238307	502	Prostaglandin synthesis	CAC AGA GCA CGG CAT CAA AAA GGA CAC CAT CGG ACA
Retinol binding protein	AF257326	477	Transport protein for vitamine A	N ATG CAR AAY TTY GAY AAR AC TGT SCN ACG CGT CTG TAT
Glucose-6-phosphatase	AF120150	532	Enzyme in the gluconeogenesis	TCA GTG GCG ACA GAA AGG TCG AGG AGA ACG TCA AGC
Transferrin	D89083	525	Transport protein for iron	ACT CTG ACA CCT GCT ATT ATG C TGC TGT AGA TGC GAT TGG
Cytochrome-C-Oxidase Subunit VIA	U83980	469	Respiratory chain (house-keeping gene)	TTA AAG GAA TGG CGT CTC TTT CTA TCT GAC TGG GAG C
Glyceraldehyde-3-Phosphate-Dehydrogenase	AB066373	462	Enzyme in the gycolysis	GTT ACA AGG GTG AGG TGA GC GGA TGA TGT TCT GGT GGG

Table A2 Spotted cDNA Fragments on the second set of slides (Chapter 3.4) (continued)

Gene Name Array 2004	Genbank Accession	Fragment Size	Protein Function	Primer Sequences (5' – 3')
Pyruvatkinase	AY113695	330	Enzyme in the gycolysis	CGC GGT AAC AAG AAG TCC TC GCA GTG CTT CCA AGA CAG AA
Thyrotropin β*	D14692	488	pituitary hormone	CTT CTG CTA CTC AAG GGA CA CAA TGA ATA GGA TAC ACC ACC
Cathepsin D	U90321	514	lysosomale enzyme	TGC TCC TTC ACA GAC ATC G CCT CAC AGC CTC CCT TAC A
Zona radiata structural protein	AF407574	442	similar to VEPβ	CAG TAC CAT TGT GGC TGT GG CTC CTC TTT CTG TAG CAT CTT GG
CYP3A45	AF267126	469	Oxigenase	GAG ATG TTT GAT GCG TTG TCC CCC GAT TAG TTG AGT TCC CAG
Albumin related protein	AF281358	402	Transport protein in the blood	GAA CCG CCT GGA GAG AGG GTC TGC GAC AGC ATC ACT AGC
Carbonyl-Reductase	AF100931	463	20β- Hydroxysteroid Dehydrogenase	ATC GGA GTG ACT GTG CTG CTT TAC ATC ATT CAA CTT TT
Cu/Zn-Superoxide-Dismutase	AF469663	437	Detoxification enzyme	GCG TGC TCA AAG GCA CC AAT GCC AAT CAC TCC ACA GG
CYP2K3	AF043551	515	Oxigenase	GTC TCC AAC ATA ATC TCA GCC AGG TTC TTC CTG TCC TCT ACC
CYP2K1v2	AF045052	488	Oxigenase	CTT TAT GCC GTC TCC AAC CCT ATG ACC CTG CTG ATC
Gonadotropin Releasing Hormone 1*	AF110992	419	involved in hormonal cascade	AGA AGG AAT AGA CCG AA TTT TAC AGG AGG TTA GTG
NADH-Dehydrogenase Subunit VI	AF125047	459	Enzyme in the respiratory chain	CGA CTC AAT CCC CGT GTT AGG GCT TGT AGC TGT TGC
Major Histocompatability Complex I	AJ251432	523	membran protein, involved in immune response	GCT GTG GGC ATC GTA AAT GTG GCA GGT CAC TGG AGA
Hemopexin	Z68112	466	Transport protein of Heme	TGA CGA CGC CGG AAA CAT GAC GCT CTG AAA GTG GAA GTA G

Table A2 Spotted cDNA Fragments on the second set of slides (Chapter 3.4) (continued)

Gene Name Array 2004	Genbank Accession	Fragment Size	Protein Function	Primer Sequences (5' – 3')
p53	AF223793	507	Tumour suppressor gene	CCC AGC CCT CCA TTT CTA GGT TCA TCC CTC CCA TAC A
CYP1A3	AF059711	489	Oxigenase	AAT AAC CCT CAC CTC AGC C GTT CAC CAA GCC CAA CAG
VEP γ	AF231708	465	acellular component of the oocyte envelope	AGA CTG CCC TAT GAC TGG A TAT CTG GGA TTG GCG TTT A
3β-Hydroxysteroid-Dehydrogenase	S72665	513	involved in steroidogenesis	AAA TGT CCG ACC ACA ACT CA TTC CGA ATC CCG TCT CCC
11β-Hydroxylase (CYP11B)	AF179894	508	involved in steroidogenesis	ACC TCT TCC GCT TCG C TCT TGC CCA CGC TAC CC
Androgen Receptor	AB012095	471	intracellular receptor for androgens	AAT GAC CTG GGA CCT AAT GTC CTC CAT TGT TTC TCC
Aryl hydrocarbon translocator	U73841	514	promotes oxygen-independent stabilization of hypoxia-inducible factor-1alpha	GAC GAG GAA GCA GAC AAT CAG GCA TCT GTG GAA GTG
Glucagon	U19914	499	increases blood glucose levels	ACT CCC TGG CTG GTG TTC CTT TTC CCT GAG GTT TTG
Monoaminooxydase*	L37878	513	neurological enzyme	ACT GTG GCA CTA TGG TGA T AGA AGG TGG TGA CAA ACG
β-actin	AJ438158	540	part of the cytoskeleton	GTC AGG CAG CTC GTA GCT CT CTG ACC CTG AAG TAC CCC ATT
Vasa*	AB032566	535	Germ cell determination	GAA CGC ACA ATG GTC TTT TAT CAG TGG AGG CAA AGG
Vitellogenin Receptor	AJ417877	450	oocyte-specific member of the low-densiby lipoprotein receptor supergene family	CCC TGG GAT CTG TAG TCA AGA ACT TTC CGT TTG GTG
Cholesterol-Side-Chain-Cleavage P450	S57305	530	Monooxigenase	GAG GAG GGT AGG AGC CA ACC AGA GTC CCA CAA GG
Gonadotropin Releasing Hormone Receptor*	AJ272116	535	localized in the pituitary	GGA CGC CAT TTG GAA CAT C GAC CAC GAA GGA CAT CAC A
VEP α	AF231706	490	acellular component of the oocyte envelope	CAA CAG ATA CCC TAC ACC AAA TAA TCA GTG TCT CAA CGG AAG

Table A2 Spotted cDNA Fragments on the second set of slides (Chapter 3.4) (continued)

Gene Name Array 2004	Genbank Accession	Fragment Size	Protein Function	Primer Sequences (5' – 3')
Glucokinase	AF053331	544	glucose homeostasis	ACA GGG TGG TGG ACG AGA ACT GCC GAG ATA AGT GCC G
Glutamate-Decarboxylase Isoform 65*	AF503210	469	present in GABA neurons	TTC CAG CAG GAC AAG CAG T GTC CCA GTC GCT CAA TCT C
Phosphoenolpyruvat-Carboxykinase	AF246149	400	Enzyme in gluconeogenesis	CCT GGC TGG ACT GTG GA CCC CGA AGA TGA TGG CA
CYP2K5	AF151524	521	Oxigenase	GGC AAT GAA TTA CGC TGT CT AAG GCC TGA CGA CTT CCT AT
Steroidogenic Acute Regulatory Protein	AB047032	534	sterol transfer protein for cholesterol	CGC TGG CAT CTC CTA CA CGA ACA CTA ACG AAG TCC C
Glutamate-Dehydrogenase	AJ419570	511	involved in amino acid metabolism	GGG CTA AAG CTG GTG TCA AG GCT TGT AGT CCT CCA GCT CCT
actin genomic band		540	DNA contamination control	GTC AGG CAG CTC GTA GCT CT CTG ACC CTG AAG TAC CCC ATT
growth hormone 1 intron C	AF005923	469	DNA contamination control	TCC ATG ATG CAA GAT TCC AA CCA CCG ATT AGA CAG TGA TGG
Poly A		50	Hybridization control	

* fragments (provided by T. Mommsen) were included for interest even though they are not specific to hepatocytes

Table A3 Results of the OD Measurements after cDNA labelling reaction (Chapter 3.4)

Exposure	Cy dye	cDNA amount [µg]	Incorporation [%]	base/dye ratio	pmol of dye incorporated
0.5% EtOH	3	0.3	13.4	27.9	35.5
10^{-7}M E_2	5	0.24	12.5	30.5	26.3
0.5% EtOH	5	0.2	4.5	86.7	7.8
10^{-7}M E_2	3	0.12	30.2	11.6	32.7
0.5% EtOH	3	1.17	7.5	50.6	76.5
10^{-7}M EE_2	5	0.28	6.2	63.6	14.8
0.5% EtOH	3	0.76	13.1	28.4	87.3
10^{-7}M EE_2	5	0.6	10.9	35.2	56.0
0.5% EtOH	5	0.56	5.2	75.4	24.6
10^{-7}M EE_2	3	1.1	14.8	25.2	134.4

Selbständigkeitserklärung

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.

Leipzig, 05.11.2004

- Anja Schreer -

Lebenslauf

Name:	Anja Schreer
Geburtsdatum und Ort:	12.02.1975 in Pritzwalk
Promotion	
seit 01.09.2001	Doktorandin am UFZ – Umweltforschungszentrum Leipzig-Halle GmbH, im Department Zelltoxikologie unter Betreuung von Frau Dr. Kristin Schirmer und Betreuung von Prof. Dr. Günter Vollmer, Institut für Zoologie, Professur für Molekulare Zellphysiologie und Endokrinologie, TU Dresden
Thema der Doktorarbeit:	„Application of rainbow trout primary hepatocytes to study gene expression due to estrogenic compounds"
	Die Doktorarbeit war Teil des Projektes: „Chip-Detekt – Entwicklung eines DNA-Chip zur Detektion hormonartiger Wirkungen in der Umwelt" gefördert durch das Sächsische Landesamt für Umwelt und Geologie (Az: 56-8811.61/91)
Studium	
seit 21.02.2001	Diplom-Biologin
April 2000 – Feb. 2001	Diplomarbeit am IZW – Institut für Zoo- und Wildtierforschung in Berlin unter der Betreuung von Dr. Martin Dehnhard und Prof. Dr. Andreas Elepfandt, Institut für Zoologie, AG Sinnesbiologie, HU Berlin
	„Das Huhn als Modellorganismus für nicht-invasives Monitoring von Stresshormonen bei Vögeln"
Okt. 1994 - Feb. 2001	Studium der Biologie an der Humboldt-Universität zu Berlin
Hauptfach	Tierphysiologie
weitere Schwerpunkte	Biochemie, Ökologie, Paläontologie
WS 1998/99	Studium an der Universität Wien im Rahmen des Sokrates Austauschprogrammes
Ausbildung	
1993 – 1994	Ausbildung zur Hotelfachfrau in Cuxhaven
Schule	
1990 – 1993	Besuch der EOS in Pritzwalk, Abschluß Abitur
1981 – 1990	Besuch der POS in Sadenbeck

UFZ-Umweltforschungszentrum
Leipzig-Halle GmbH
Permoserstraße 15, 04318 Leipzig
Internet: www.ufz.de