Chemical mixtures and interactions with detoxification mechanisms and biomarker responses in fish

Johanna Gräns

Akademisk avhandling för filosofie doktorsexamen i naturvetenskap, inriktning biologi, som med tillstånd från Naturvetenskapliga fakulteten kommer att offentligt försvaras fredagen den 30 januari 2015 kl. 10.00 i föreläsningssalen, Zoologen, Institutionen för biologi och miljövetenskap, Medicinaregatan 18A Göteborg

UNIVERSITY OF GOTHENBURG

Department of Biological and Environmental Science
Faculty of Science

2015

Published papers are reproduced with permission from the publisher Elsevier.

Cover illustration by Jesper Halling

Published by the Department of Biological and Environmental Science, University of Gothenburg, Sweden.

© Johanna Gräns, 2015

ISBN 978-91-628-9261-6

http://hdl.handle.net/2077/37781

Printed by Kompendiet, Göteborg (http://www.kompendiet.se)

Dissertation Abstract

Several classes of anthropogenic chemicals are present as mixtures in the aquatic environment. However, information of how wildlife species, including fish, are affected by exposures to chemical mixtures is limited. Chemicals interactions, due to shared elimination pathways or receptor interactions, can result in possible adverse outcomes in animals. This thesis investigates how detoxification mechanisms are affected by exposure to chemicals alone or in mixtures. Cytochrome P450 (CYP) enzymes and efflux pumps have key roles in the detoxification and elimination pathways of many structurally different chemicals and are therefore targets for chemical interactions. Induction of the CYP1A isoform and the egg-yolk precursor vitellogenin in fish are two established biomarkers used to assess exposures to aromatic hydrocarbons and estrogens in the environment.

This thesis focuses on regulation of these biomarkers, with emphasis on regulation and function of CYP1A, in fish or cultured fish liver cells exposed to different classes of chemicals alone and in mixtures. This thesis shows that structurally different chemicals can interact on regulation of CYP1A gene expression and/or on catalytic function. A synergistic mixture effect on the CYP1A biomarker response was demonstrated in the *Poeciliopsis lucida* hepatocellular carcinoma cell line, upon combined exposure to β-naphthoflavone (BNF) and different azoles. The synergistic mixture effect is caused by inhibition of the CYP1A catalytic function. An antagonistic mixture effect on the vitellogenin biomarker was demonstrated in primary cultures of rainbow trout hepatocytes upon combined exposure to BNF, which activates the aryl hydrocarbon receptor (AhR), and the synthetic estrogen 17α-ethinylestradiol, which activates the estrogen receptor (ER). The antagonistic mixture effect is caused by an inhibiting AhR-ER cross-talk. A cross-talk between the AhR and the pregnane-X-receptor (PXR) was also suggested in livers of the PCB-resistant killifish population from the New Bedford Harbor, Massachusetts, USA. These fish have reduced AhR-CYP1A signaling but respond to exposure to PCBs in the laboratory by increased PXR, CYP3A and efflux pump mRNA levels.

This thesis shows that these biomarkers are affected, in fish or in fish liver cells, exposed to chemical mixtures. This can have adverse effects on their detoxification mechanisms and can also lead to misinterpretation of biomarker data in biomonitoring programs in the aquatic environment.

Keywords:
Fish, AhR, CYP1A, PXR, chemical mixtures, chemical interaction, biomarker

Populärvetenskaplig sammanfattning

Vår miljö är ständigt utsatt för en mängd olika kemikalier varav många hamnar i våra sjöar, hav och vattendrag. Kemikalier som påträffats i miljön kommer bland annat från läkemedel, hygienprodukter, bekämpningsmedel, industrikemikalier samt vid förbränning av fossila bränslen. Det sker riskbedömningar för kemikalier och deras gränsvärden men dessa är baserade på ett ämne i taget och tar inte hänsyn till kemikaliernas eventuella blandningseffekter. Dessutom kan de biomarkörer som används i miljöövervakningar för att påvisa förekomsten av kemikalier i miljön vara påverkade av blandningar. Det kan i sin tur leda till feltolkning av data och missbedömningar av förekomsten av miljögifter. Detta är ett problem eftersom de flesta kemikaler i miljön förekommer i form av olika blandningar och det saknas kunskap om blandningseffekter. För fisk som lever och förökar sig i en miljö där kemikalieblandningar förekommer kan detta leda till negativa effekter på hälsa och fertilitet. Cytokrom P450 (CYP) enzymer har en viktig roll i avgiftningsprocessen där fettlösliga kemikalier omvandlas i kroppen till mer vattenlösliga ämnen som kroppen sedan lättare kan göra sig av med. Denna process är utsatt för eventuella interaktioner mellan olika kemikalier vilket kan påverka avgiftningsförmågan.

Denna avhandling består av fyra artiklar som på olika sätt belyser fiskars avgiftningsförmåga när de blir utsatta för olika kemikalier var för sig eller i blandningar. De olika artiklarna visar också hur biomarkörer i fisk påverkas när de utsätts för kemikalieblandningar. Fokus i denna avhandling är CYP1A. Ett enzym som är viktig i avgiftningen av många miljögifter. Fiskar har normalt låga nivåer av CYP1A men om de blir utsatta för aromatiska kemikalier så ökar nivåerna kraftigt. Det är därför CYP1A-ökningen rutinmässigt används som en biomarkör för att uppskatta förekomsten av aromatiska kemikalier i miljön. De olika studierna i avhandlingen visar bland annat att flera kemikalier minskar CYP1A aktiviteten vilket innebär en ökad risk att ackumulera kemikalier till hälsofarliga nivåer. Dessutom påverkar kemikalieblandningar CYP1A som biomarkör, vilket kan leda till missbedömning av förekomsten av aromatiska kemikalier i miljön. Detta riskerar ge en felaktig bild över hur vår miljö mår.

List of publications

The thesis is based on the following papers, which are referred to by their Roman number in the text:

I. Wassmur, B., **Gräns, J.**, Norström, E., Wallin, M., Celander, M.C. 2013. Interactions of pharmaceuticals and other xenobiotics on key detoxification mechanisms and cytoskeleton in *Poeciliopsis lucida* hepatocellular carcinoma, PLHC-1 cell line. *Toxicology in Vitro*, 27: 111-120

II. **Gräns, J.**, Johansson, J., Michelová, M., Wassmur, B., Norström, E., Wallin, M., Celander, M.C. Synergistic and antagonistic interactions between different azoles and β-naphthoflavone on the CYP1A biomarker in a fish cell line. *Submitted for publication in Aquatic Toxicology*

III. **Gräns, J.**, Wassmur, B., Celander, M.C. 2010. One-way inhibiting cross-talk between arylhydrocarbon receptor (AhR) and estrogen receptor (ER) signaling in primary cultures of rainbow trout hepatocytes. *Aquatic Toxicology*, 100: 263-270

IV. **Gräns, J.**, Wassmur, B., Fernández-Santoscoy, M., Zanette, J., Woodin, B.R., Karchner, S.I., Nacci, D.E., Champlin, D., Jayaraman, S., Hahn, M.E., Stegeman, J.J., Celander, M.C. Regulation of pregnane-X-receptor, CYP3A and P-glycoprotein genes in the PCB-resistant killifish (*Fundulus heteroclitus*) population from New Bedford Harbor. *Aquatic Toxicology*, *in press* http://dx.doi.org/doi:10.1016/j.aquatox.2014.12.010

Abbreviation list

ABC	ATP-binding cassette
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BFCOD	7-Benzyloxy-4-(trifluoromethyl)-coumarin-O-debenzyloxylase
BLAST	Basic local alignment search tool
BNF	β-Naphthoflavone
CYP	Cytochrome P450
DRE	Dioxin response element
E_2	17β-Estradiol
EDC	Endocrine disrupting chemical
EE_2	17α-Ethinylestradiol
ER	Estrogen receptor
EROD	7-Ethoxyresorufin-*O*-deethylase
GST	Glutathione *S*-transferases
Hsp90	Heat shock protein 90
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistant protein
NBH	New Bedford Harbor
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
Pgp	P-glycoprotein
PHAH	Planar halogenated aromatic hydrocarbons
PLHC-1	*Poeciliopsis lucida* hepatocellular carcinoma
PXR	Pregnane-X-receptor
RACE	Rapid amplification of cDNA ends
RXR	Retinoid-X-receptor
SC	Scorton Creek
VTG	Vitellogenin
WHO	World Health Organization
XRE	Xenobiotic response element

Table of contents

1. Introduction 1
1.1. Our chemical society 1
1.2. Chemicals in the aquatic environment 2
1.2.1. Polycyclic aromatic hydrocarbons (PAHs) 3
1.2.2. Polychlorinated biphenyls (PCBs) 4
1.2.3. Pharmaceuticals 5
1.2.4. Azoles 6
1.3. Detoxification mechanisms 7
1.3.1. Fish and chemical exposures 7
1.3.2. Biotransformation in fish 8
1.3.3. The cytochrome P450 (CYP) gene superfamily 9
1.3.4. The CYP1A subfamily in fish 10
1.3.5. Activation and action of AhR in vertebrates 11
1.3.6. Cytoskeleton and CYP1A induction 12
1.3.7. The CYP3A subfamily 13
1.3.8. The efflux pumps 14
1.4. Chemical resistance in fish 15
1.4.1. New Bedford Harbor (NBH) 15
1.4.2. Chemical resistance mechanisms 16
1.5. Endocrine disrupting chemicals (EDCs) 16
1.5.1. Estrogens and estrogenic substances in the environment 17
1.5.2. Androgens and anti-estrogens in the environment 19
1.6. Application of biomarkers to assess chemical exposures 19
1.6.1. Induction of CYP1A as a biomarker for aromatic hydrocarbons 20
1.6.2. Induction of VTG as a biomarker for estrogenic chemicals 21
1.7. Mixture effects 21
1.7.1. Chemical interactions 21
1.7.2. Interactions of CYP1A enzyme activity 23
1.7.3. Receptor interactions 24
2. Scientific aim 26
2.1. Overall aim 26
2.2. Specific aims 26
3. Materials and methods 27
3.1. Experimental animals and cell models 27
3.1.1. Rainbow trout 27
3.1.2. Killifish 28
3.1.3. Guppy 28
3.1.4. Primary cell cultures 28
3.1.5. *Poeciliopsis lucida* hepatocellular carcinoma (PLHC-1) cell line 28
3.2. Substances tested 29
3.3. Methods 30

3.3.1. Induction and inhibition ... 30
3.3.2. Quantification of mRNA levels ... 31
3.3.3. Measurement of 7-ethoxyresorufin-O-deethylase (EROD) activity ... 32
3.3.4. Measurement of 7-benzyloxy-4-(trifluoromethyl)-coumarin-O-debenzyloxylase (BFCOD) activity ... 32
3.3.5. Quantification of efflux pump activity ... 33
3.3.6. CYP1A protein quantification using Western blot ... 33
3.3.7 Immunocytochemistry ... 33
3.3.8 Isolation and gene analyzes ... 33
3.3.9 Phylogenic analysis ... 34
4. Findings and discussion ... 35
4.1. Regulation and function of CYP1A (by classical and non-classical AhR ligands) ... 35
4.1.1. Azoles and steroidogenic CYP enzymes ... 35
4.1.2. Azoles and induction of CYP1A ... 36
4.1.3. Azoles and inhibition of CYP1A ... 37
4.1.4. EDCs and effects on CYP1A ... 39
4.1.5. Microtubules and CYP1A induction ... 40
4.2. Function and regulation of CYP3 in the PLHC-1 cell line ... 42
4.2.1. Identification of a PLHC-1 CYP3 gene ... 42
4.2.2. Regulation and function of CYP3B ... 43
4.3. Function and regulation of efflux pumps ... 44
4.3.1. Efflux inhibition ... 44
4.3.2. Efflux activation ... 44
4.3.3. Efflux induction ... 45
4.4. Mixture effects ... 46
4.4.1. Synergistic effects on CYP1A after co-exposure to azoles and BNF ... 47
4.4.2. AhR-ER cross-talk in fish ... 48
4.4.3. Mixture effects on biomarker responses ... 50
4.5. Chemical resistance ... 50
4.5.1. Chemical resistance mechanisms: Putative receptor cross-talk? ... 51
5. Conclusions and future perspectives ... 52
Acknowledgments ... 54
6. References ... 56

1. INTRODUCTION

A large amount of chemicals are in use and many of these will eventually reach the aquatic environment as complex mixtures. Despite this, there is inadequate knowledge of how mixture exposures affect aquatic organisms, such as fish. Mixture effects can be additive, synergistic or antagonistic and can cause unwanted health effects in fish. This thesis addresses effects on the detoxification pathway in fish exposed to chemical mixtures.

1.1 Our chemical society

Chemicals are all around us and are a part of our daily life and our wealth. A higher demand and consumption in today's society has led to an increased chemical production. The world-wide chemical production is over 400 million tons per year, which is an increase by over 4 000% over the last 50 years (the Swedish Environmental Protection Agency, 2013). Our chemical society is diverse with over 100 000 different chemicals, and approximately 30 000 of these are in daily use (the Swedish Environmental Protection Agency, 2013). In addition, the uses of pharmaceuticals steadily increase. In Sweden, the number of approved pharmaceuticals had reached about 12 000 in year 2013 (Medical Products Agency Sweden, 2013), and the total sale of pharmaceuticals during 2012 was almost 3.9 million €. Man-made chemicals have improved health, well-being and life-quality for many individuals, but there is an increase in certain diseases and other adverse outcomes that are correlated to increased exposures to chemicals. Increased frequencies of cardiovascular diseases, cancer, Parkinson's disease, obesity, diabetes and reproductive diseases are some examples which can be linked to increased exposures to chemicals (reviewed in World Health Organization (WHO), 2006; Bergman et al., 2013). In addition, an increased production and consumption of chemicals results in an increased emission of chemicals to the environment, with increased adverse effects on exposed biota as a consequence. The European Commission has established several directives and legislations in order to deal with the problems and to protect the environment within the European Union. The REACH legislation (Registration, Evaluation, Authorisation and Restriction of Chemicals) aims to protect human health

and the environment. In addition, certain classes of chemicals, depending on the area of use e.g. pharmaceuticals and biocides, are regulated by specific legislations with different requirements on the risk assessment. Furthermore, in order to protect the environment, directives such as the Water Framework Directive and the Marine Strategy Framework Directive have been implemented (European Commission, 2010). However, chemical risk-assessments are typically based on single chemical exposure experiments and the current legislations and directives usually do not consider mixture toxicity. This is problematic as chemicals most often end up as mixtures in the environment.

1.2 Chemicals in the aquatic environment

Most chemicals eventually end up in the aquatic environment which has become a global concern as they pose a major threat to the aquatic ecosystems. Several classes of anthropogenic (man-made) chemicals including pharmaceuticals, personal-care products, pesticides, polychlorinated biphenyls, dioxins, polycyclic aromatic hydrocarbons, nanoparticles and metals have been detected. There are numerous sources from which pollutants may enter into the environment and these can be classified as point-sources or diffuse sources. Point-source exposures normally result in a direct release into the environment and include effluents from industry, sewage treatment plants, accidental spills, land disposal sites and resource extractions. Diffuse sources normally result in wide-spread releases into the environment from agriculture, atmospheric depositions and urban runoffs (Holt, 2000; Ritter et al., 2002). Fate and effects of chemicals in the environment can differ due to the chemical properties and environmental conditions. A chemical can be dissolved or form a complex with particles and can also undergo transformation to non-toxic or more toxic forms by microbial, chemical and photolytic actions (Ritter et al., 2002). The amount of a chemical which is available for uptake by organisms is defined as the bioavailability of the chemical (Holt, 2000).

Ultimately, the aquatic environment is subjected to continuous exposures to different classes of chemicals from different uses and sources. This thesis

focuses on effect of environmentally relevant chemical classes including aromatic hydrocarbons, pharmaceuticals and azoles in fish.

1.2.1 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a large, diverse chemical group consisting of substances with three or more benzene rings (**Figure 1**). Although some PAHs occur naturally in the environment, the impact and adverse effects associated with this class of compounds are mainly due to anthropogenic activity, generating PAHs from combustion processes (pyrogenic PAHs) and fossil fuel production (petrogenic PAHs) (Neff et al., 2005; Hylland, 2006). Consequently, the sources of PAH in the aquatic environment are diverse and include precipitation, oil spills, petroleum- and smelter industries, transportations, runoff from roads and diverse other coastal activities (Hylland, 2006; de Hoop et al., 2011). In the aquatic environment, PAHs can bind to particles and organic materials or remain dissolved, depending on their physical properties, which can make them more or less persistent and bioavailable (Neff et al., 2005; Hylland, 2006). In this thesis, the effects of PAH-type chemicals alone or in mixtures are investigated on detoxification mechanisms and biomarker responses in fish.

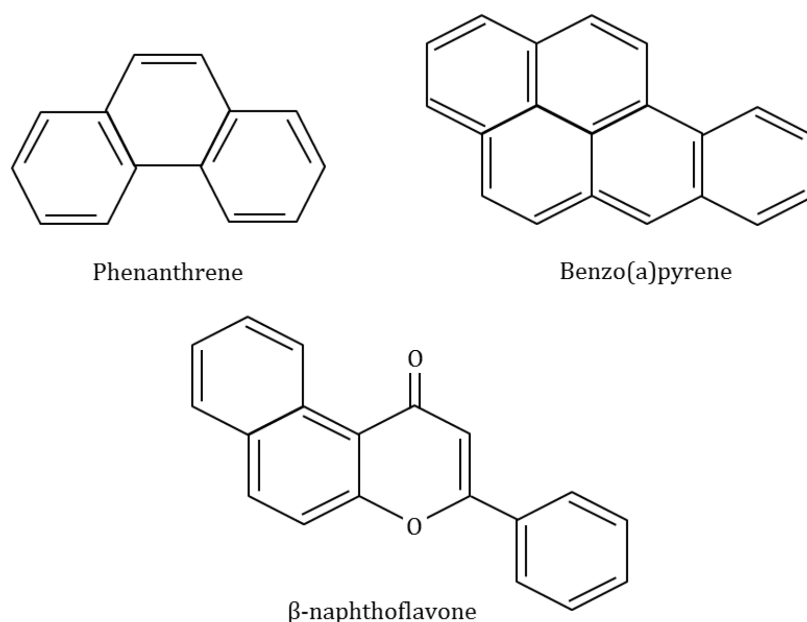

Figure 1. Phenanthrene and benzo(a)pyrene are examples of polycyclic aromatic hydrocarbons (PAHs) and β-naphthoflavone is a PAH-like model substance.

1.2.2 Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are synthetic compounds consisting of a biphenyl which is substituted with one to ten chlorine atoms. There are 209 different PCB congeners, depending on how many of carbon atoms that are chlorinated on the biphenyl and at which position (*ortho*, *para* and *meta*) (**Figure 2**) (Safe, 1992; Beyer and Biziuk, 2009; Erickson and Kaley, 2011). PCBs have been widely used in production of electric components (e.g. transformers and condensers), as plasticizers and as pesticide extenders (Safe, 1992; Beyer and Biziuk, 2009; Weber et al., 2011) to name a few areas of use. The PCBs are highly persistent and are still present in the environment even though manufacturing of PCBs was banned in many countries in the late 1970s. The environmental PCB contaminations are widespread and PCBs are routinely detected in atmospheric, water, sediment and soil samples. Industrialized areas, particularly those with former production plants, are highly contaminated with PCBs (Beyer and Biziuk, 2009). In addition, there are ongoing new emissions of PCBs to the aquatic environment, mainly by leakage from landfills (Sundberg et al., 2005; Weber et al., 2011). The PCBs bind strongly to organic particles in the water column, atmosphere, sediment and soil. Highly chlorinated non-*ortho* substituted and mono-*ortho*-substituted PCBs (i.e. PCBs with no or one chlorinated at *ortho*-positions) are more prone to binding to organic particles, increasing their resistance to microbial degrading. Therefore, they are more often detected in soil and sediment (Beyer and Biziuk, 2009; Erickson and Kaley, 2011). The PCBs are persistent and lipophilic and can therefore bioaccumulate and also biomagnify through the food chain, which result in higher concentrations in organisms at higher trophic levels. This includes fish and piscivorous predators (Burreau et al., 2006; Houde et al., 2008; Ruus et al., 2012). Another great concern is the ability of PCBs to volatilize into the atmosphere where they can be transported over long distances. For example, the polar areas which have no PCB production or use are contaminated by long-distance PCBs as a result of the global distillation process (Ritter et al., 2002; Gouin et al., 2004). In this thesis, effects of a non-*ortho*-substituted (PCB 126) and an *ortho*- substituted (PCB 153) PCB are investigated on detoxification mechanisms in a PCB resistant fish population from North America.

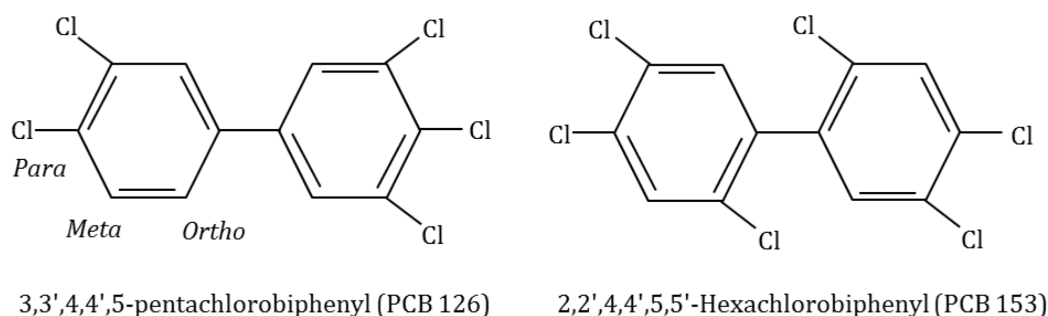

Figure 2. Examples of non-*ortho* (PCB 126) and *ortho* (PCB 153) substituted PCB congeners.

1.2.3 Pharmaceuticals

The production and use of pharmaceuticals is steadily increasing and pharmaceutical contamination of the aquatic environment has received increased attention during the last two decades (Halling-Sørensen et al., 1998; Heberer, 2002; Fent et al., 2006; Kümmerer, 2009; Boxall et al., 2012). There are several reports of detectable concentrations of pharmaceuticals in sewage treatment effluents, groundwater, and drinking water, ranging from ng L^{-1} to µg L^{-1} (reviewed in: Kümmerer, 2009; Corcoran et al., 2010). However, higher concentrations of up to mg L^{-1} of some pharmaceuticals have been reported in the environment surrounding pharmaceutical production plants (Larsson et al., 2007; Fick et al., 2009). The concentration found for some pharmaceuticals in rivers and lakes in these areas even exceed their therapeutic plasma levels (Schulz et al., 2012). The concentrations found in the environment are often described as being below the concentration limit to cause acute toxic effects. However, pharmaceuticals are designed to have effects at low concentrations and to be biologically active with specific molecular targets. Therefore, non-target organisms can be subjected to potentially harmful levels of pharmaceuticals in their natural habitat during their whole life cycle. In this thesis, effects of different classes of pharmaceuticals alone and in mixtures on detoxification mechanisms and fish biomarker responses are investigated.

1.2.4 Azoles

Azoles are five-membered heterocyclic nitrogen-containing compounds with at least one other non-carbon atom (**Figure 3**). Azoles, including imidazoles, triazoles and benzimidazoles, represent a relatively diverse class of chemicals with a broad use. Azoles were mainly developed as antifungal agents for use in agriculture and horticulture and as antifungal drugs for clinical uses (Vanden Bossche et al., 2003; Chambers et al., 2014). In addition to the antifungal effect, azoles are used as biocides, e.g. in marine paints to prevent fouling (Dahlström et al., 2000), as anti-ulcer drugs in humans, and azoles are used in veterinary medicine to treat certain parasite infections in domestic animals (Campbell, 1990; Koop and Arnold, 1991). Several azoles have been detected in rivers, sewage treatment effluents and sludge as well as in fish caught outside sewage treatment plants (Kreuger, 1998; Castillo et al., 2000; Lindberg et al., 2010; Fick et al., 2011; Belenguer et al., 2014; Moschet et al., 2014). In this thesis, the effects of azoles alone or in mixture were investigated on detoxification mechanisms and biomarker responses in fish

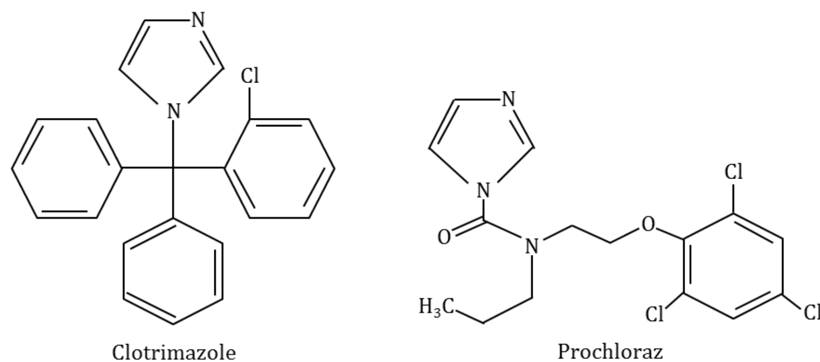

Figure 3. Clotrimazole and prochloraz are examples of imidazoles.

1.3 Detoxification mechanisms

1.3.1 Fish and chemical exposures

Numerous environmental pollutants are capable of causing a variety of toxic effects in organisms including neurotoxicity, reproductive and developmental (teratogenic) effects and carcinogenicity (reviewed in: Carney et al., 2006; White et al., 2007; Cheshenko et al., 2008; King-Heiden et al., 2012; Budinsky et al., 2014). The extent to which a chemical accumulates in a fish depends chiefly on four processes; absorption, distribution, metabolism and excretion (**Figure 4**) (Kleinow et al., 2008; Nichols et al., 2009). Chemical uptake in fish can occur through gills and skin from the ambient water or through the gastro-intestinal tract from the diet. The main organ for detoxification metabolism is the liver, but this also takes place in the intestine, gills and kidney. Chemicals and/or their metabolites are excreted via the gills, as biliary products in feces from the intestine or as urinary products from the trunk kidney.

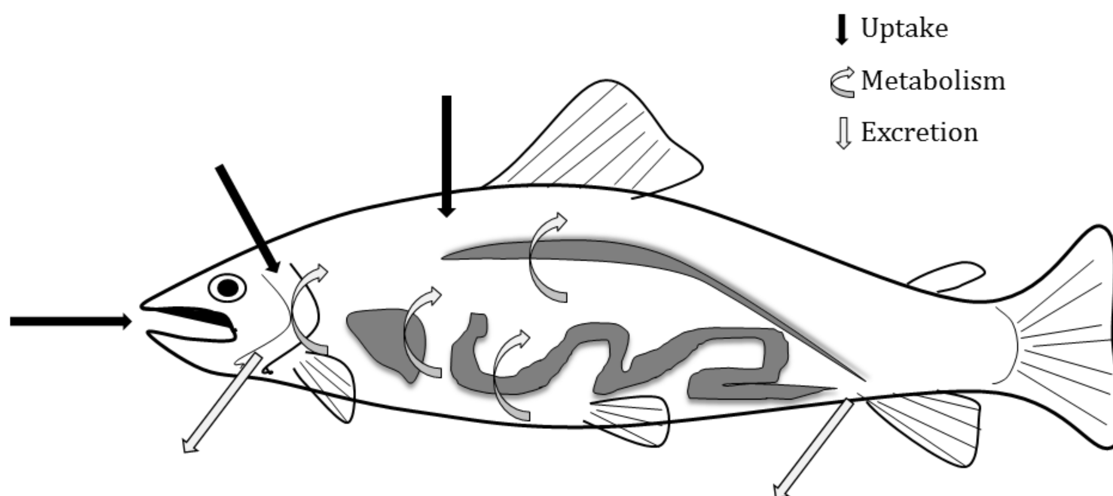

Figure 4. Gills, skin and diet are the primary routes of chemical uptake. The main organs for detoxification and metabolism are liver, intestine, gills and kidney. Chemicals are excreted via gills, intestine and kidney.

This thesis focuses on the effects of single chemicals and chemical mixtures on the detoxification system in fish liver cells *in vitro* and fish liver and gills *in vivo*. The detoxification system for lipophilic organic substances in a fish involves enzymes which metabolize a lipophilic chemical to a more

hydrophilic metabolite via biotransformation. Biotransformation proceeds in two phases, phase 1 and phase 2 (**Figure 5**). In concert with biotransformation enzymes, there are specific transporters that actively pump chemicals or their metabolites out of the cell. This process is called efflux, and takes place in either phase 0 or phase 3 (**Figure 5**).

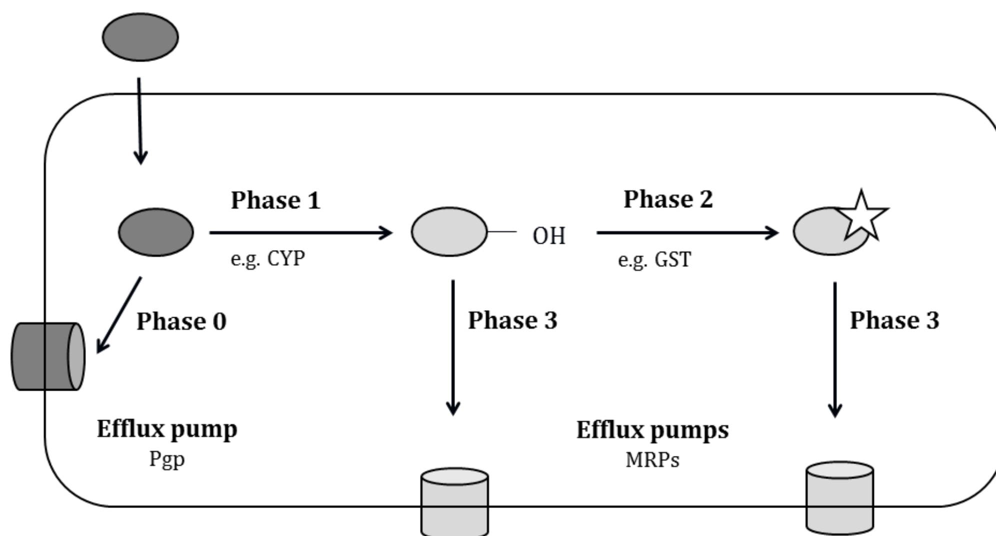

Figure 5. Phase 1 mediated by CYP enzymes and phase 2 biotransformation reactions mediated by glutathione-*S*-transferases (GST) enzymes and efflux mediated by P-glycoprotein (Pgp) and multidrug-resistant proteins (MRPs) transporters.

1.3.2 Biotransformation in fish

Many chemicals are lipophilic and are therefore not easily excreted. Biotransformation results in increased elimination rates of lipophilic organic chemicals and can thereby prevent bioaccumulation to toxic concentrations. The biotransformation process involves transformation of lipophilic xenobiotics (foreign chemicals) as well as endobiotics (endogenous chemicals) to more hydrophilic metabolites (Nebert, 1994). These hydrophilic metabolites can more easily be excreted via the bile, urine or gills in fish. Biotransformation can also generate metabolites that are more reactive (i.e. toxic) than the parent chemical. Hence, biotransformation affects bioaccumulation, persistence, distribution and the toxicity of a chemical in fish (Kleinow et al., 1987; Livingstone, 1998; Schlenk et al., 2008). The biotransformation processes are catalyzed by a range of enzymes that act in concert. In the phase 1 reaction, a functional

group, usually a –OH, -COOH, -SH or $–NH_2$, is attached to the substrate via oxidation, reduction or hydrolysis, which increases the water solubility of the chemical. Therefore, phase 1 enzymes are also called functionalization enzymes as they introduce a functional group into the chemical and thereby make it more active so it can be further metabolized in phase 2. Examples of biotransformation enzymes involved in the phase 1 reactions are CYP monooxygenase, flavoprotein monoxygenase, monoamine oxidase, epoxide hydrolase and reductase. The phase 2 reactions involve conjugation of the phase 1 metabolites with a polar endogenous molecule (i.e. glutathione, glucuronic acid or sulphate). These conjugation reactions are catalyzed by glutathione-*S*-transferases, UDP-glucuronosyltransferases or sulphonyl-transferases. The phase 2 enzymes are also called conjugation enzymes. The phase 2 reactions further increase the water solubility of a chemical and thus its ability to be more efficiently excreted (Livingstone, 1998; Schlenk et al., 2008). The majority of the phase 1 reactions are catalyzed by CYP enzymes in both mammals and fish. This thesis focuses on CYP reactions in phase 1 biotransformation in fish.

1.3.3 The cytochrome P450 (CYP) gene superfamily

The CYP enzymes are membrane-bound hemoproteins found in every class of organisms, eukaryotes as well as prokaryotes (Ortiz de Montellano, 2010). A CYP protein was first described in 1958 by Klingenberg as a pigment that absorbs light at 450 nm and was therefore named P450. The protein was further characterized, as belonging to the group of cytochromes, in 1962 by Omura and Sato and was subsequently named cytochrome P450. There are now over thousand known CYP sequences which are, based on the sequence identity, grouped into clades, gene families (e.g. CYP1, CYP2 and CYP3), subfamilies (e.g. CYP1A and CYP1B) and individual genes (e.g. CYP1A1 and CYP1A2) (Guengerich, 2001; Nelson, 2006). Mammalian and fish CYP enzymes can largely be divided into non-inducible anabolic enzymes and inducible catabolic enzymes. The majority of CYP mediated reactions results in a hydroxylated metabolite (Guengerich, 2001; Schlenk et al., 2008; Ortiz de Montellano, 2010). The overall redox reaction can be described, where the substrate is referred to as R-H and the hydroxylated metabolite as R-OH, as follows:

$$R\text{-}H + O_2 + NADPH + H^+ \longrightarrow R\text{-}OH + H_2O + NADP^+$$

The anabolic CYP enzymes are typically constitutively expressed and catalyze biosynthesis of endogenous lipophilic compounds such as steroids, growth factors and fatty acids (Guengerich, 2001; Nebert and Russel, 2002). The catabolic CYPs catalyze the breakdown of lipophilic endobiotics as well as lipophilic xenobiotics and the key enzymes in this metabolism are members of CYP1 to CYP4 families (Nebert and Russel, 2002). Expression of the catabolic CYP enzymes is induced via specific receptors and is commonly up-regulated as a response to exposure to a variety of lipophilic substances. Induction of CYP metabolism generally results in enhanced elimination that prevents bioaccumulation of xenobiotics to toxic concentrations. However, CYP metabolism can also cause adverse outcome by formation of activated and more toxic metabolites and increased production of reactive oxygen species (Schlezinger et al., 1999; Guengerich, 2001; Denison et al., 2011). Accelerated CYP metabolism can also lead to depletion of hormones, lipophilic vitamins and therapeutic drugs that can result in adverse health effects (Guengerich, 2001; Nebert and Russel, 2002).

In fish, multiple CYP forms have been identified. For example, in zebrafish (*Danio rerio*), 94 CYP genes have been identified (Goldstone et al, 2010). The zebrafish CYPs are divided into two major functional groups, those involved in biosynthesis (CYP5 to CYP51) and those involved in xenobiotic metabolism (CYP1 to CYP4) (Schlenk et al., 2008; Goldstone, 2010). This thesis focuses on function and regulation of the CYP1A and CYP3A subfamilies in fish, which catalyze breakdown of lipophilic xenobiotics, such as PAHs, PCBs, pharmaceuticals and azoles as well as lipophilic endobiotics, such as fatty acids and prostaglandins.

1.3.4 The CYP1A subfamily in fish

One of the most studied CYP form in fish is the CYP1A subfamily. The CYP1A enzyme has a key role in the metabolism of many environmental aromatic contaminants. Furthermore, induction of CYP1A is used to assess exposure to PAHs and planar halogenated aromatic hydrocarbons (PHAHs) in biomonitoring programs in the aquatic environment (Stegeman and Hahn,

1994; Hahn, 2002). This is further discussed in section 1.6.1. Expression of the CYP1A gene is normally low in fish which have not been exposed to aromatic contaminants. However, exposure to a wide range of structurally diverse aromatic chemicals can induce the expression of the CYP1A gene, a process mediated via the aryl hydrocarbon receptor (AhR). Many of the CYP1A inducers are also substrates to the CYP1A enzyme, which means that these chemicals induce their own biotransformation and elimination (Guengerich, 2001).

This thesis focuses on CYP1A with respect to its role in the detoxification of xenobiotics. The induction of CYP1A by different classes of chemicals is addressed in all papers (**Paper I-IV**) of the thesis. In addition, effects of chemical exposures on AhR-CYP1A signaling are specifically addressed in **Paper II**, **Paper III** and in **Paper IV**.

1.3.5 Activation and action of AhR in vertebrates

The AhR is a ligand-activated transcription factor belonging to the basic-helix-loop-helix family of proteins. The expression of a large battery of genes, such as CYP1A, is controlled by AhR and mediates a wide range of biological responses (Schmidt and Bradfield, 1996; Denison et al., 2011). The best characterized high-affinity AhR ligands are PHAHs and PAHs. However, a wide range of structurally diverse chemicals can bind to and activate AhR-mediated responses (Stegeman and Hahn, 1994; Denison et al., 2011). Several studies in mammals have shown that AhR has an endogenous role in normal physiology and development processes (Reviewed in McMillan and Bradfield, 2007). In addition, endogenous ligands such as the tryptophan photoproduct 6-formylindolo[3,2-*b*]carbazole, have been identified in several species (Rannug et al., 1987; Jönsson et al., 2009; Laub et al., 2010). The mechanism of AhR induction has been extensively studied in mammalian systems. The classical mechanistic model of AhR action is illustrated in **Figure 6**. In the absence of a ligand, the AhR is located in the cytosol as a multi-protein complex with chaperone proteins including heat shock protein 90 (Petrulis and Perdew, 2002; Beischlag et al., 2008; Denison et al., 2011). Upon ligand-binding, the AhR undergoes a conformational change which facilitates translocation of the complex into the nucleus, followed by dimerization with the aryl

hydrocarbon receptor nuclear translocator (ARNT) and displacement of associated proteins. The AhR:ARNT complex has high affinity for dioxin/xenobiotic response elements (DRE/XRE) in the promoter region of target genes, such as the CYP1A gene, and activates their transcriptions (Beischlag et al., 2008; Denison et al., 2011). In fish, both AhR (Abnet et al., 1999; Karchner et al., 1999; Tanguay et al., 1999; Hansson et al., 2003) and ARNT (Pollenz et al., 1996; Powell et al., 1999; Tanguay et al., 2000) have been identified and the signaling pathway is believed to be similar to that described in mammals. In addition, the AhR can exert other actions via receptor cross-talk with other receptors, such as the estrogen receptor (ER). This is further discussed in this thesis in section 1.7.3.

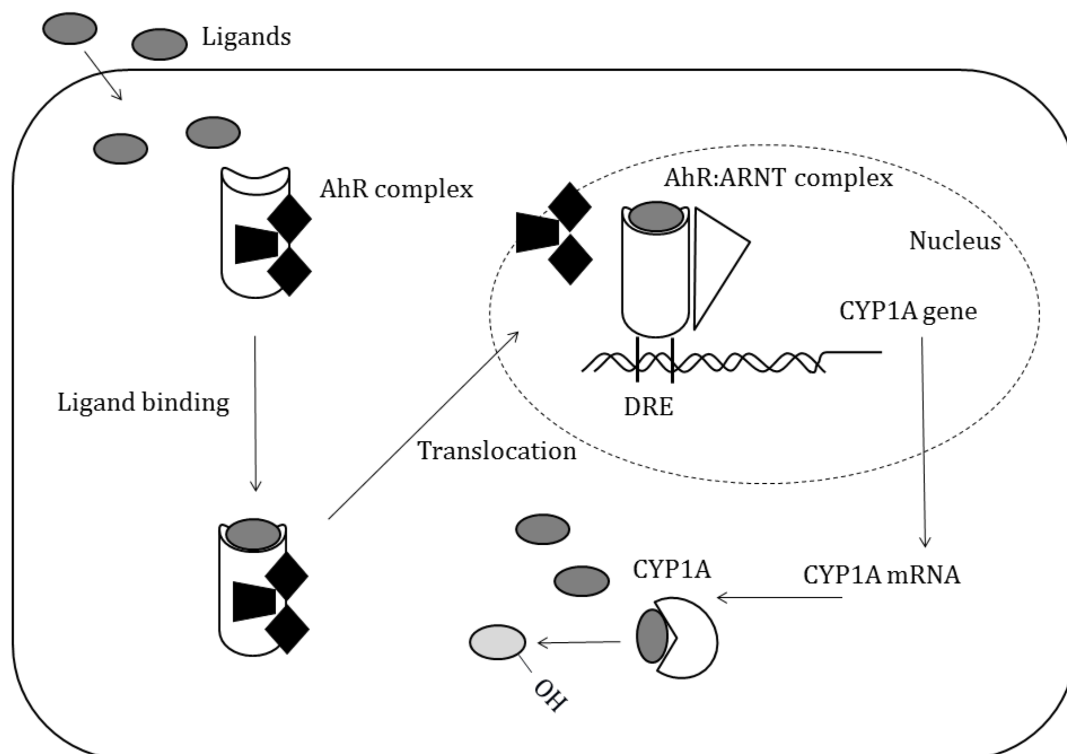

Figure 6. The mechanism of AhR mediated CYP1A induction. Ligand binding to the cytosolic AhR complex results in translocation into the nucleus. In the nucleus dimerization with ARNT and displacement of associated proteins resulting in binding of AhR:ARNT complex to DRE which activates transcription of the CYP1A gene.

1.3.6 Cytoskeleton and CYP1A induction

The cytoskeleton is essential for cell survival and cellular functions and it has key roles in cell division, cell motility, cell adhesion, intracellular

transports and cellular signaling. The cytoskeleton comprises three types of structures; microtubules, intermediates filaments and actin filaments. Microtubules are involved and important in e.g. cell proliferation and in the cellular transports of organelles and other components (Dumontet and Jordan, 2010). It has been suggested that microtubules enable translocation of the glucocorticoid receptor and androgen receptor from the cytosol to the nucleus upon activation (Harrell et al., 2004; Thadani-Mulero et al., 2012). The mechanism by which the AhR is translocated from the cytoplasm into the nucleus is not yet clear, but a microtubule-dependent mechanism has been proposed (Dvořák et al., 2006). In **Paper I**, the effect of pharmaceutical exposure on microtubule and actin filament integrity was investigated. Furthermore, **Paper II** addresses the question of the importance of an intact microtubule network for CYP1A induction.

1.3.7 The CYP3A subfamily

The CYP3A isoform is the predominant CYP form in the liver in both fish and mammals (Celander et al., 1996a; Thummel and Wilkinson, 1998; Hegelund and Celander, 2003). The CYP3A enzymes have broad substrate specificities and therefore can metabolize a large number of structurally diverse lipophilic chemicals, including pharmaceuticals and endogenous steroids (Thummel and Wilkinson, 1998; Guengerich, 1999; Nebert and Russel, 2002). Many of the compounds that are substrates to the CYP3A enzyme can also induce CYP3A gene expression. The CYP3A gene expression in mammals is mediated via the pregnane-X-receptor (PXR), which belongs to the nuclear receptor superfamily. It functions as a ligand-activated transcription factor. Activation of PXR by ligand binding results in a dimerization with retinoid-X-receptor (RXR), following binding to the PXR response element in the CYP3A gene (Kliewer et al., 1998; Pascussi et al., 1999). The PXR is often referred to as being promiscuous due to its wide ligand specificity. Glucocorticoids (such as dexamethasone), anti-glucocorticoids (such as pregnenolone-16α-carbonitrile) and macrolide antibiotics (such as rifampicin) are classical mammalian PXR ligands and prototypical CYP3A inducers (Kliewer, 2003).

Compared with mammals, less is known about the PXR signaling pathway and CYP3A regulation in fish. However, CYP3A induction is most likely also

mediated via PXR activation in fish (Wassmur et al., 2010; Bainy et al., 2013). The coding sequence for PXR have been identified in a few fish species including rainbow trout (*Oncorhynchus mykiss*), green spotted pufferfish (*Tetraodon nigroviridis*) and zebrafish (Wassmur et al., 2010; Krasowski et al., 2011; Bainy et al. 2013). Besides, CYP3A proteins and coding sequences have been identified in several fish species (Celander et al., 1996a; McArthur et al., 2003). Compared with mammals, CYP3A genes in fish are less inducible, so that exposure to classical PXR ligands, such as dexamethasone, pregnenolone-16α-carbonitrile or rifampicin, has no effect or only slightly increases CYP3A expression (Celander et al., 1996b; Bresolin et al., 2005; Wassmur et al., 2010; Bainy et al., 2013). In this thesis, the PXR-CYP3A signaling pathway in fish liver cells is addressed in **Paper I** and **Paper IV.**

1.3.8 The efflux pumps

Efflux pumps are membrane-bound proteins belonging to the superfamily ATP-binding cassette (ABC) transporter proteins, which actively pump xenobiotic or their metabolites out of the cell by a process called efflux (Leslie et al., 2005; Luckenbach et al., 2014). The ABC genes are divided into subfamilies (e.g. ABCA, ABCB and ABCC) and individual genes (e.g. ABCB1 and ABCC1). The ABCB1 gene codes for P-glycoprotein (Pgp) and ABCC genes code for multidrug resistant proteins (MRPs). The MRPs and Pgp are key players in defense and detoxification of xenobiotics in vertebrates. In the detoxification reactions, un-metabolized xenobiotics are mainly transported by Pgp which is sometimes referred to as phase 0 reactions. Metabolites of phase 1 and 2 reactions are mainly exported out of the cells by MRPs, sometimes referred to as phase 3 reactions in the detoxification pathway (**Figure 4**) (Leslie et al., 2005). The presence of Pgp (Sturm et al., 2001; Cooper et al., 1999; Hemmer et al., 1995) as well as MRPs (Luckenbach et al., 2014) has been demonstrated in several fish species and these pumps have been suggested to have a role in the defense and detoxification of xenobiotics also in fish (Luckenbach et al., 2014). A decreased accumulation of xenobiotics has been found in organisms living in highly polluted areas, a phenomenon described as multixenobiotic or multidrug resistance (Kurelec, 1992). On the contrary, many chemicals are able to inhibit the efflux pumps and thereby impair their functions.

Chemicals inhibiting the efflux pumps are sometimes referred to as chemosensitizers as they can lead to an increased uptake of other chemicals which can cause adverse effects as a result of increased bioaccumulation of xenobiotics (Smital and Kurelec, 1998).

The knowledge about the occurrence and function of MRPs and Pgp in fish is limited compared with that in mammals. In this thesis, regulation of MRPs and Pgp has been investigated in **Paper I** and in **Paper IV**. In **Paper I** interference with the efflux function was also studied.

1.4 Chemical resistance in fish

Killifish or mummichog (*Fundulus heteroclitus*) populations which are able to live and reproduce in highly PCB contaminated areas have been reported in several studies in North America (Van Veld and Westbrook, 1995; Nacci et al., 1999; Bello et al., 2001). These populations have adaptated to tolerate toxic levels of PCBs (Nacci et al., 2010). One area which is heavily polluted with PCBs is the New Bedford Harbor in Massachusetts, USA, which was the target site investigated in **Paper IV**.

1.4.1 New Bedford Harbor (NBH)

The New Bedford Harbor (NBH) is an area with a long history of waterfront industries which have caused severe contamination problems (Weaver, 1984; Nelson and Bergen, 2012). For instance, from the 1940's to the 1970's, PCBs were used by manufactures of electronic parts, resulting in a release of PCBs to the harbor (Weaver, 1984). In 1983, NBH was classified as a superfund site and cleanup of the harbor started in 1994 (Nelson and Bergen, 2012). The cleanup process is still ongoing and is estimated to continue for several more years before completed (U.S. Environmental Protection Agency, 2014). The cleanup has resulted in decreased PCB concentrations, although relatively high PCB levels are still detected (Nelson and Bergen, 2012). In addition to monitoring of the sediment, tissue concentrations of PCBs in resident killifish populations have also been measured as well as studies on genetic adaptations in these fish populations (Nacci et al., 2010; **Paper IV**).

1.4.2 Chemical resistance mechanisms

Extremely high tissue levels of *ortho*-substituted PCBs as well as non-*ortho* substituted PCBs have been detected in NBH killifish compared with killifish from reference sites (Lake et al., 1995; Black et al., 1998). The non-*ortho* substituted PCBs normally have high affinity to AhR and induce CYP1A gene expression in fish (Stegeman and Hahn, 1994). However, one characteristic of the NBH killifish is low CYP1A protein levels and activity as well as reduced CYP1A induction by exposure to typical inducers (Nacci et al., 1999; Bello et al., 2001). Activation of AhR and CYP1A mediated biotransformation can generate reactive oxygen species (Schlezinger et al., 1999). Reduced CYP1A capacity can therefore be advantageous in certain situations, such as for fish that reside in heavily polluted environments. Previous studies in killifish suggest that the mechanism of toxicity resistance and absence of CYP1A induction is likely to be at the AhR level (Clark et al., 2010; Whitehead et al., 2010; Oleksiak et al., 2011). In addition, some *ortho*-substituted PCBs are mammalian PXR ligands and induce the expression of CYP3A genes in mammals (Jacobs et al., 2005; Al-Salman and Plant, 2012; Gährs et al., 2013). It was not previously known if killifish from NBH respond to *ortho*-substituted PCBs and whether the PXR-signaling pathway is affected. Therefore, the expression of PXR, CYP3A and Pgp genes in fish from NBH, with disrupted AhR signaling was investigated in **Paper IV**. In addition, the response to exposure to an *ortho*-substituted PCB and a non-*ortho*-substituted PCB was studied in the laboratory and compared with that in killifish from the relatively uncontaminated reference site, Scorton Creek (SC) in Massachusetts (**Paper IV**).

1.5 Endocrine disrupting chemicals (EDCs)

Over the last twenty years, EDCs have received increased attention and been recognized as a potential hazard for human and the environment. This has led to vigorous efforts by international bodies such as the European Commission and WHO to initiate research programs and development of new strategies concerning EDCs. The WHO definition of an EDC is “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact

organism, or its progeny, or (sub)populations" (Bergman et al., 2013). A large number of chemicals within different classes have been identified as endocrine disrupters including for example persistent organic pollutants (including certain PCBs), pharmaceuticals, personal care products, pesticides, flame retardants and plasticizers (Bergman et al., 2013). There are numerous reports on the effect of EDCs in fish, including disruption on sexual development and reproduction. The EDCs can act by mimicking or blocking natural hormone functions and thereby affect many hormonal systems including the estrogen signaling system.

1.5.1 Estrogens and estrogenic substances in the environment

Estrogens are steroid hormones which play an important role in a wide range of physiological processes including reproduction. Estrogenic actions are mediated via the ER that controls the expression of a number of genes (Gao and Dahlman-Wright, 2011). The sex steroid hormone 17β-estradiol (E_2) is the most common estrogen, with important regulatory function in female vertebrates, including fish. Synthesis of E_2 occurs primarily in the theca and granulosa cells in the ovarian follicles. The final step in estrogen synthesis is the conversion of testosterone to E_2, which is catalyzed by the aromatase (CYP19) enzymes (Nagahama, 1994; Bondesson et al., 2014). The E_2 hormone is essential for a wide range of processes including gene expression of an egg-yolk protein precursor called vitellogenin (VTG) in the liver. The VTG synthesis in the liver is a process known as vitellogenesis and is followed by VTG transport via the blood to the developing ovaries and uptake of VTG into the oocytes (Nagahama, 1994; Arukwe and Goksøyr, 2003), where it serves as a source of nutrition during embryonal development (Arukwe and Goksøyr, 2003). This is illustrated in **Figure 7**.

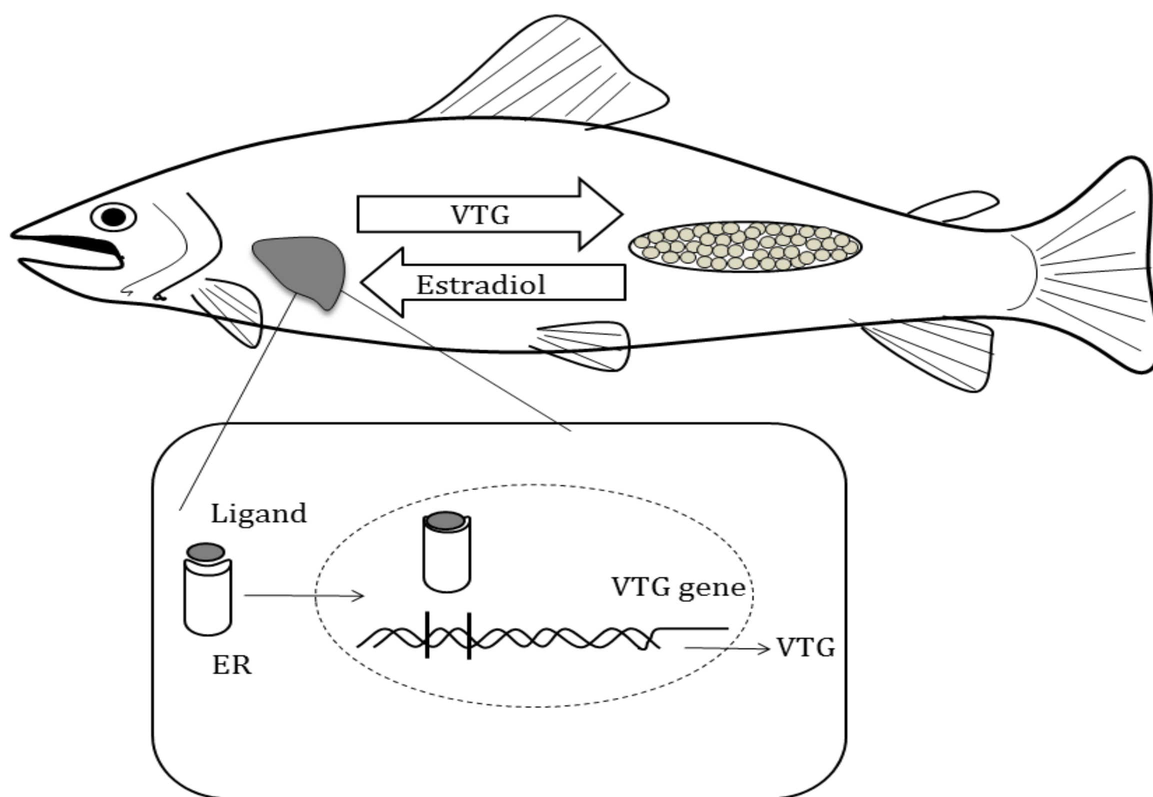

Figure 7. The vitellogenin (VTG) synthesis. The E_2 hormone binds to the estrogen receptor (ER) followed by activation and transcription of VTG genes. The VTG proteins are transported via the blood and taken up by the oocytes.

In addition to naturally produced estrogen hormones, compounds that mimic estrogens and act as ER agonists are referred to as estrogenic chemicals. These belong to the diverse group of EDCs. The ER agonists comprise synthetic estrogens, such as 17α-ethinylestradiol (EE_2), and estrogenic substances such as bisphenol A. Bisphenol A is not classified as an estrogen, but can act as an ER agonist. The VTG synthesis occurs naturally in mature female fish prior spawning. However, VTG synthesis can also be induced in male and juvenile fish following exposure to ER agonists, leading to ER activation. Therefore, VTG induction in male and/or juvenile fish is used to assess prior exposures of fish to estrogens and estrogenic substances in the aquatic environment, and this biomarker is further discussed in section 1.6.2.

One EDC substance of particular concern is the synthetic estrogen, EE_2, which is the active substance in contraceptive pills of combination type.

This EDC is extremely potent and is present in effluent from sewage treatment plants together with other estrogens such as E_2. Feminization, including intersex and presence of egg-yolk proteins in male fish, has been reported in fish near sewage treatment plants (Purdom et al., 1994; Larsson et al., 1999; Jobling et al., 2002) and in fish exposed to waste water effluents (Lange et al., 2011; Harris et al., 2011). In a large-scale field experiment, a fathead minnow (*Pimephales promelas*) population in a Canadian experimental lake collapsed and nearly reached extinction after exposure to an environmentally relevant concentration of EE_2 (Kidd et al., 2007).

1.5.2 Androgens and anti-estrogens in the environment

Masculinization of female fish exposed to pulp and paper mill effluents have been reported, likely as a result of androgen exposures (Cody and Bortone, 1997; Larsson and Förlin, 2002; Parks et al., 2001). Several azoles have also been shown to exert anti-estrogenic effects. For example, masculinization has been demonstrated in zebrafish exposed the fungicide prochloraz (Baumann et al., 2014). In addition, exposure to prochloraz decreases estradiol plasma levels, egg-yolk proteins and reduces the number of eggs laid by female fathead minnows (Ankley et al., 2005)

1.6 Application of biomarkers to assess chemical exposures

Many countries apply monitoring programs, including chemical monitoring and biological monitoring, to determine levels of contaminants in the environment. In biological monitoring, biomarkers are frequently used to assess the occurrence of and exposure to a chemical (Peakall and Walker, 1994; Van der Oost et al., 2003). Peakall and Walker (1994) defined biomarker as "a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also, of toxic effect". Biomarkers can be used at several levels from molecular to organismal and can, especially when used at the molecular level, be applied as an early-warning system (Peakall and Walker, 1994). So what is a good biomarker? Van der Oost et al. (2003) proposed six criteria that should be available or established regarding important information for a biomarker:

1) The assays should be reliable, relatively cheap and easy to perform.
2) Well defined baseline data should be available to distinguish between natural variability and variance due to presence of chemicals.
3) The response should be sensitive to exposure.
4) There must be knowledge about confounding factors and their impact on the biomarker response.
5) The biomarkers toxicological significance should be established
6) There should be a well-known relationship between the biomarker response and pollutant exposure.

1.6.1 Induction of CYP1A as a biomarker for aromatic hydrocarbons

Induction of CYP1A in fish is commonly used as a biomarker to assess exposures to organic aromatic contaminants such as certain PAHs and PHAHs in the environment (Stegeman and Hahn, 1994; Whyte et al., 2000; Sarkar et al., 2006; Schlenk et al., 2008). The levels of CYP1A in unexposed fish are normally low, but can be highly induced by exposure to AhR agonists. Induction of CYP1A is therefore an excellent biomarker routinely used to assess exposures to AhR agonists. The CYP1A induction in environmental monitoring is most commonly assessed by measuring the CYP1A enzyme activity in a reaction called ethoxyresorufin-*O*-deethylase (EROD) activity using ethoxyresorufin as a diagnostic substrate. There are several advantages of using EROD activity as the biomarker measure. The assay is relative cheap, sensitive and gives a fast quantifiable response upon exposure (Whyte et al., 2000). In addition to enzyme activity, CYP1A induction can also be determined by measuring CYP1A -protein- or mRNA levels. The presence of AhR ligands in fish and their environment is highly correlated to the induction of CYP1A (Goksøyr and Förlin, 1992; Hahn et al., 2002). However, fish may be exposed to mixtures of different classes of chemicals which may affect the CYP1A induction response and/or the CYP1A mediated EROD activity in different manners. Thus, the EROD activity is not always correlated with the presence of aromatic hydrocarbons. The effects of chemicals and mixtures on the CYP1A biomarker response are addressed in **Papers I**, **II** and **III**.

1.6.2 Induction of VTG as a biomarker for estrogenic chemicals

As mention earlier, the estradiol levels in males and juveniles are normally too low to induce VTG gene expression. However, the VTG gene can be induced upon exposure to estrogens and estrogenic chemicals and can therefore be used as a biomarker of exposure to ER agonists in the aquatic environment (Sumpter and Jobling, 1995; Hutchinson et al., 2006). There are several properties which make VTG an excellent biomarker, such as high specificity to ER agonists and the highly sensitive response. In addition, there are well established assays for VTG levels available for a wide range of different fish species (Hutchinson et al., 2006). Induction of VTG can be determined by measuring VTG protein levels in plasma or VTG mRNA levels in liver. The effects of chemicals and mixtures on the VTG biomarker response are addressed in **Paper III**.

1.7 Mixture effects

In the fields of toxicology and ecotoxicology, chemical mixtures have recently received greater attention. With an increasing knowledge about mixture toxicity it has been recognized that a chemical mixtures can result in increased adverse responses at concentrations where each chemical of the mixture has no effect. However, while resent research has advanced the understanding of mixture toxicity (Walter et al., 2002; Faust et al., 2003; Backhaus et al., 2004; Hass et al., 2007; Christiansen et al., 2009; Celander, 2011; Kortenkamp and Faust, 2010; Hadrup et al., 2013; Orton et al., 2014), regulatory protocols for assessing the safety of chemicals are still based on single chemical exposure testing (Backhaus and Faust, 2012; Kienzler et al., 2014).

1.7.1 Chemical interactions

In situations of mixture exposure, toxicodynamic or toxicokinetic mechanisms can result in different types of chemical interactions (**Figure 8**). When different chemicals have the same mode of action, toxicodynamic interactions occur. When a chemical alters the uptake, distribution, metabolism and excretion of another substance, this is known as toxicokinetic interaction (Scripture and Figg, 2006; Celander, 2011).

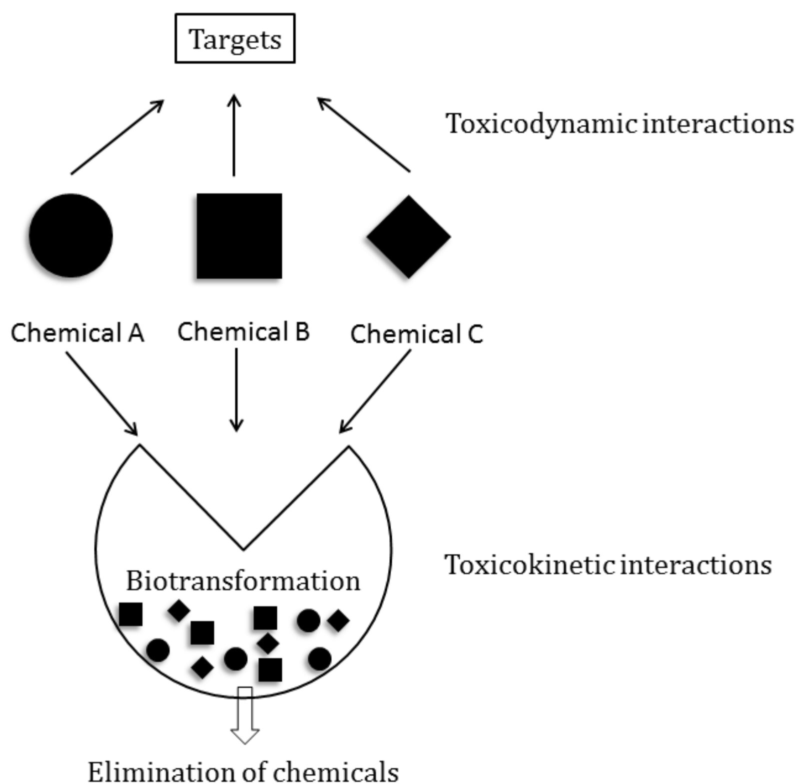

Figure 8. Chemical interactions. Toxicodynamic interactions occur when different chemicals have the same or opposite mode of action. Chemicals sharing the same elimination pathway can cause toxicokinetic interactions.

Interactions between chemicals at the receptor, transporter or enzyme level can result in synergistic or antagonistic responses (**Figure 9**) (Scripture and Figg, 2006; Celander, 2011). Interactions affecting the metabolic and excretion pathway may result in a decreased clearance and increased accumulation of harmful chemicals and vice versa, and are well known problems in human toxicology. As an example, the effect of contraceptive pills can be insufficient in women who are also taking herbal medicines containing St. John's wort (*Hypericum perforatum*). This antagonistic toxicokinetic interaction is linked to increased CYP3A4 expression induced by St. John's wort resulting in enhanced clearance of the contraceptive (Huang et al., 2004). Another example of synergistic toxicokinetic interactions is inhibition of CYP3A4 by grapefruit juice causing decreased CYP3A4-dependent clearance of the antihistamine terfenadine and bioaccumulation to lethal concentrations in humans

(Huang et al., 2004). Studies in fish also show increased toxicity and adverse responses by chemical mixtures (Hermens et al., 1985; Hasselberg et al., 2008; Laetz et al., 2009; Galus et al., 2013). However, toxicokinetic interactions are less established in the aquatic environment and the knowledge of the effects in fish are limited. In addition, exposure to mixtures can also affect biomarker responses in fish and for accurate interpretations of biomonitoring data, the mixture toxicity has to be addressed (Celander, 2011). This thesis focuses on toxicokinetic interactions in fish that are affecting CYP-mediated biotransformation and ABC transporter mediated efflux of chemicals (**Papers I-IV**).

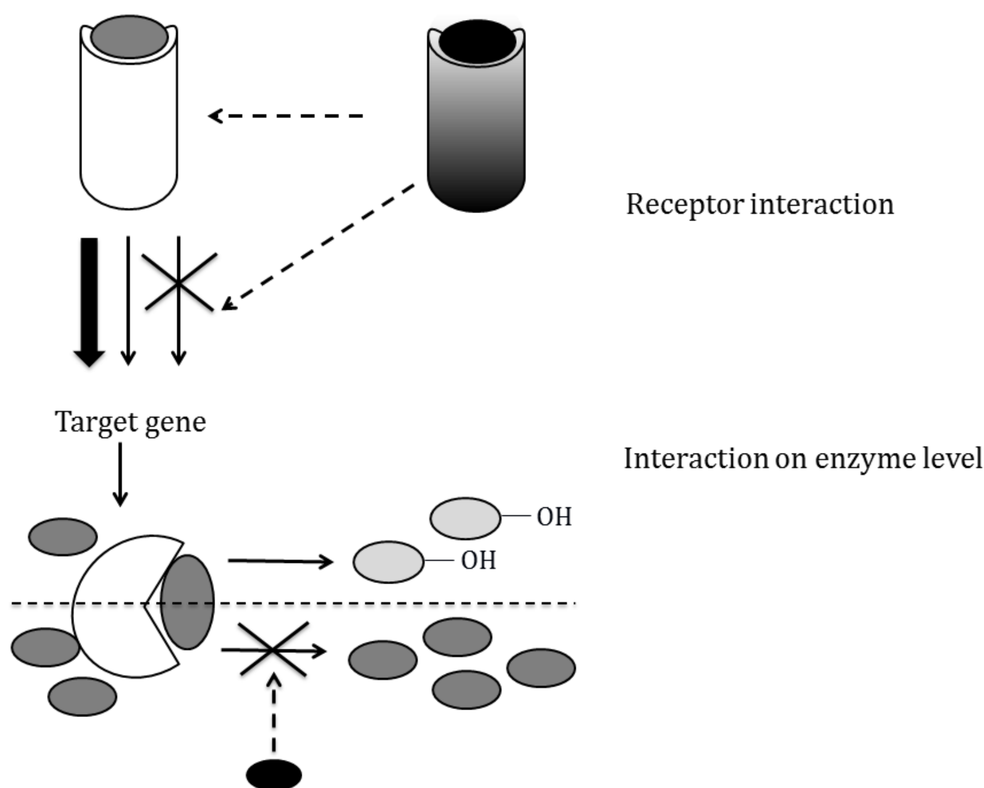

Figure 9. Chemical interaction can take place on the receptor level and/or the enzyme level. A receptor can inhibit or stimulate the activity for another receptor and thereby affect the transcription of the target gene. Interactions on enzymes by inhibition of the activity can result in decreased elimination rate and increased bioaccumulation of other chemicals.

1.7.2 Interactions of CYP1A enzyme activity

There are several types of enzyme inhibition. Competitive inhibition occurs when there is competition for the active sites (substrate binding site) on the enzyme. In un-competitive inhibition, an inhibitor can only bind to an

already substrate-bound enzyme. A mixture of competitive and un-competitive inhibition where a chemical can bind to the enzyme regardless of whether a substrate is bound or not is referred to as mixed inhibition. Non-competitive inhibition is a form of mixed inhibition. Regardless, chemical interactions caused by CYP1A enzyme inhibition can result in an increased bioaccumulation of chemicals that are metabolized by the CYP1A enzymes. There are several chemicals which are able to inhibit the CYP1A activity. For example, antifungal azoles are designed to inhibit fungal CYP51-mediated 14α-demethylase activity which is required in the ergosterol biosynthesis (Henry and Sisler, 1984; Vanden Bossche et al., 1995). However, the inhibition of CYP51 by azoles is non-specific and can inhibit multiple CYP forms including catabolic CYP enzymes in fish. Thus, e.g. in gizzard shad (*Dorosoma cepedianum*) the imidazole clotrimazole acts as a non-competitive CYP1A inhibitor and results in bioaccumulation of benzo(a)pyrene when co-exposed with clotrimazole (Levine et al., 1997). In addition, the imidazole ketoconazole is a potent non-competitive CYP1A and CYP3A inhibitor in Atlantic cod (*Gadus morhua*) liver (Hasselberg et al., 2005). In rainbow trout, co-exposure to ketoconazole and the synthetic estrogen EE_2 results in an increased estrogenic response compared with exposure to EE_2 alone due to inhibition of CYP1A and CYP3A activities (Hasselberg et al., 2008). In this thesis, interactions on CYP1A enzyme activity were addressed in **Paper I**, **Paper II** and **Paper III**.

1.7.3 Receptor interactions

Interactions at receptor levels, cross-talk between AhR and other nuclear receptors can affect gene expressions and result in either reduced or enhanced gene induction. Cross-talk between AhR and other receptors in fish have been demonstrated in several studies. The possible mechanisms underlying cross-talk are various. For instance, sharing of ARNT co-factor, sharing of co-activators or sharing response element are some suggested mechanisms (Pascussi et al., 2004; 2008; Nakata et al., 2006). Interactions between AhR and glucocorticoid receptor were suggested in a study using a fish cell line, *Poeciliopsis lucida* hepatocellular carcinoma (PLHC-1), where the dioxin-dependent induction of CYP1A was potentiated by a cortisol and the synthetic glucocorticoids dexamethasone and prednisone (Celander et al., 1996b). Interactions between AhR and the hypoxia inducible factor have

also been suggested, where hypoxia inducible factor sequester ARNT from AhR thereby limiting the AhR signaling pathway (Prasch et al., 2004; Fleming et al., 2009). The most well studied receptor interaction is that between AhR and ER, which has been studied in mammalian tumor cells. Inhibitory AhR-ER cross-talks are described in human breast cancer cells and rodent mammary tumors, where AhR agonist activity has been shown to have endocrine disruptive effects (Safe et al., 2000; Matthews and Gustafsson, 2006). Conclusions on whether the ER-AhR cross-talk is reciprocal or one-directional varies (Klinge et al., 1999; Matthews and Gustafsson, 2006).

Compared with mammals, the AhR-ER interaction in fish is less studied. However, there are studies in fish describing the anti-estrogenic effect of an AhR agonist (Anderson et al., 1996; Bemanian et al., 2004; Navas and Segner, 2000; Mortensen and Arukwe, 2007; Mortensen et al., 2006). In addition, a reciprocal inhibitory ER-AhR cross-talk has also been suggested in rainbow trout and salmon hepatocytes, where studies report down-regulated CYP1A levels by ER agonists (Mortensen et al., 2006; Mortensen and Arukwe, 2007). In addition, activating reciprocal AhR-PXR cross-talk has been described in zebrafish. In that study, PXR activation resulted in up-regulated AhR2 and CYP1A while AhR2 activation caused up-regulation of PXR, CYP3A and CYP2 (Kubota et al., 2014). Cross-talk between AhR and ER was investigated in **Paper III** and putative cross-talk between AhR and PXR is discussed in **Paper IV**.

2. SCIENTIFIC AIM

2.1 Overall aim

The main objective of this thesis was to increase the knowledge of chemical mixtures on detoxification mechanisms and on biomarker responses in fish.

2.2 Specific aims

- To clarify how structurally diverse substances alone and in mixtures interact with function and regulation of CYP1A, CYP3A and efflux pumps in fish.
  (**Paper I**)

- To elucidate the importance of intact microtubules network for CYP1A induction.
  (**Paper I** and **Paper II**)

- To investigate possible cross-talk between AhR-ER signaling in fish. (**Paper III**)

- To identify PXR in killifish and study PXR, CYP3A and Pgp mRNA levels and responsiveness to exposures to *ortho*- and non-*ortho* substituted PCBs in the laboratory in a killifish with disrupted AhR-CYP1A signaling.
  (**Paper IV**)

3. MATERIALS AND METHODS

3.1 Experimental animals and cell models

In this thesis, fish or fish cell lines have been used as model systems. Fish are the most diverse vertebrate group and there are about 30 000 extant species (www.fishbase.org). As pointed out earlier, many chemicals end up in the aquatic environment and fish are good models for investigating chemical exposures on for example biomarker responses and detoxification mechanisms. Furthermore, many fish species are economically important and important protein source for humans.

In vitro systems have become important tools in aquatic toxicology as a complement to *in vivo* studies and are useful for toxicity screening. Furthermore, from an animal ethical perspective *in vitro* models are favorable and are in line with the 3R-principle, which call for refine, reduce and replace the use of laboratory animals. Today, there are a wide range of different cell models and immortalized cell lines available.

3.1.1 Rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) was used in **Paper III** to obtain primary cultures of hepatocytes as described in section 3.1.4. Rainbow trout are easily obtained from local hatcheries and are easy to maintain in laboratory conditions. It is a commonly used teleost model species for research in ecotoxicology, including studies on detoxification mechanisms, EDCs and pharmaceuticals. Furthermore, there are multiple gene sequences available. Rainbow trout was therefore a suitable species to work with in order to investigate cross regulatory interactions between the AhR and ER signaling pathway in **Paper III**.

3.1.2 Killifish

Killifish (*Fundulus heteroclitus*) is a non-migrating species that inhabits the Atlantic coast-line of North America. When studying responses to environmental changes, killifish is a good model as it is stationary and with broad distribution. In addition, they can live in highly polluted sites and have evolved tolerance to certain toxic chemicals. This has led to their common use in toxicology mechanisms studies (Burnett et al., 2007). In **Paper IV**, killifish collected from the highly polluted NBH site and the SC reference site was used to examine detoxification mechanisms in a PCB resistant population.

3.1.3 Guppy

Guppy (*Poecilia reticulata*) was used in **Paper I** to isolate a CYP3A sequence from in order to find a CYP3 transcript in a cell line derived from the related guppy species (*Poeciliopsis lucida*).

3.1.4 Primary cell cultures

In paper III primary cultures of hepatocytes from rainbow trout were used. Cells were isolated using a two-step perfusion protocol by Berry and Friend (1969) and modified for rainbow trout by Pesonen and Andersson (1991). The collagenase perfusion solution dissociates liver cells providing single cells, primarily hepatocytes. Primary cell cultures are widely used in physiological and toxicological research due to their ability to maintain several *in vivo* characteristics. There are previous studies showing that biotransformation enzymes in teleost primary cultures are maintained and stable (Segner, 1998). In addition, cells isolated from a single fish can be used to conduct several experiments and thereby reduce the number of laboratory animals in line with the 3R-principle.

3.1.5 Poeciliopsis lucida hepatocellular carcinoma (PLHC-1) cell line

The immortalized PLHC-1 cell line is derived from a liver tumor in the desert topminnow, *Poeciliopsis lucida*, was used in **Papers I** and **II**. In contrast to primary cells cultures, cell lines are normally highly proliferating and may have lost some biochemical characteristics and functions. This must be taken into account in data interpretation. However,

the PLHC-1 cell line has maintained several functions and is a suitable model for studying the detoxification system. It has a functional AhR, is able to induce CYP1A expression, has a functional glucocorticoid receptor, and possesses functional efflux activities (Hahn et al., 1993; Celander et al., 1996b; Hestermann et al., 2000; Zaja et al., 2007).

3.2 Substances tested

In this thesis, a total of 21 substances with different mode of action were studied. In **Paper I**, a screening of 18 substances was performed, mainly pharmaceuticals and model substances. In **Paper III** when studying AhR-ER interactions, the model substance β-naphthoflavone (BNF) was used as an AhR agonist, the model substance 17α-ethinylestradiol (EE_2) was used as an ER agonist and fulvestrant (ICI 182 780) was used as an ER antagonist. The AhR agonist BNF was also used in **Paper II** in combination with azoles, including the fungicide prochloraz, the anti-mycotic drug clotrimazole, the anti-ulcer drug omeprazole and the model microtubule assembly inhibitor nocodazole. Two different PCBs, the non-*ortho*-PCB 126 (dioxin-like) and the *ortho*-PCB 153 (non-dioxin-like), were used in **Paper IV**. In **Table 1** all substances used in this thesis and their known mode of action in mammals are described.

Table 1. Substances used and their modes of action in mammals.

	Substance	Mode of action in mammals	Paper
Manufacturing products	Bisphenol A	Estrogen receptor agonist	I
	PCB 126	Aryl hydrocarbon receptor agonist	IV
	PCB 153	Pregnane-X-receptor agonist	IV
Model substances	α-Naphthoflavone	Aryl hydrocarbon receptor antagonist	I
	β-Naphthoflavone	Aryl hydrocarbon receptor agonist	I, II, III
	Lithocholic acid	Pregnane-X-receptor agonist	I
	Nocodazole	Microtubule disrupter	I, II
	Pregnenolone-16α-carbonitrile	Pregnane-X-receptor agonist	I
Pesticide	Prochloraz	Fungicide	II
Pharmaceuticals	Clotrimazole	Antifungal drug	I, II
	Dexamethasone	Glucocorticoid receptor agonist	I
	Diclofenac	Non-steroidal anti-inflammatory drug	I
	Ethinylestradiol	Estrogen receptor agonist	I, III
	Fulvestrant	Estrogen receptor antagonist	I, III
	Ibuprofen	Non-steroidal anti-inflammatory drug	I
	Ketoconazole	Antifungal drug	I, II
	Omeprazole	Proton pump inhibitor	I
	Paracetamol	Analgesic drug	I
	Quinidin	Anti-arrhythmic drug	I
	Rifampicin	Macrolide antibiotic drug	I
	Troleandemycin	Macrolide antibiotic drug	I

3.3 Methods

A brief description of the methods used in the thesis work is presented here as detailed methodological descriptions are given in the four papers (**Papers I-IV**).

3.3.1 Induction and inhibition

A substance can affect enzyme activity and efflux activity, either on gene expression level or activity level. In order to analyze induction, messenger ribonucleic acid (mRNA) levels, protein levels, enzyme activities and efflux

activities were measured after certain exposure time (e.g. 6-72 hours). Induction studies were carried out both *in vivo* and *in vitro*. To analyze inhibition at CYP1A enzyme activities, the PLHC-1 cell line (**Paper I** and **II**) and rainbow trout microsomes (**Papers II** and **III**) pre-exposed to BNF, to obtain high amount of CYP1A enzymes, were used. After acute exposures to test-chemicals, CYP1A enzyme activities were measured and IC_{50} (median inhibition concentrations) were calculated. In addition, the kinetics of the inhibition of CYP1A activities by prochloraz was analyzed and plotted in either Dixon or Cornish-Bowden plots to determine type of inhibition and the inhibition constants, K_i and K_i', respectively in **Paper II.**

3.3.2 Quantification of mRNA levels

In all **Papers (I-IV)**, quantitative reverse transcriptase polymerase chain reaction (qPCR) was used to measure mRNA levels. In this fluorescence-based reaction, the amplification products during each cycle are monitored. The cycle at which the sample reaches the threshold value is negatively correlated to the mRNA level, that is, a low cycle value represents a high mRNA level in the sample (**Figure 10**). Measuring mRNA levels using qPCR gives high specificity and sensitivity. However, it gives no information on whether protein translation will follow, resulting a functional product. Measuring protein levels and/or enzyme activities as a complement to analysis of mRNA levels is advantageous.

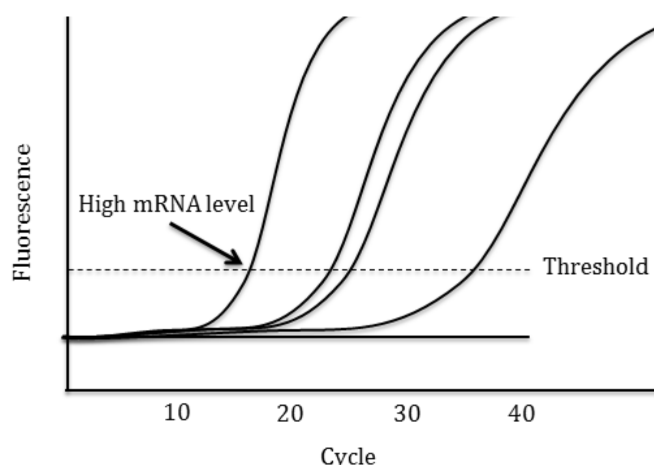

Figure 10. Amplification curves for qPCR demonstrating correlation between cycle threshold numbers and mRNA levels.

3.3.3 Measurement of 7-ethoxyresorufin-O-deethylase (EROD) activity

In **Paper I-III**, CYP1A activity was measured using 7-ethoxyresorufin as a diagnostic substrate in a reaction termed 7-ethoxyresorufin-*O*-deethylase (EROD) activity according to the protocol described in Celander et al. (1996b). The EROD activity is measured in a fluorescence-based assay in which 7-ethoxyresorufin is catalyzed by the CYP1A enzyme to a fluorescence product, resorufin. The amount of produced resorufin is measured in a fluorometer with excitation wavelength at 530 nm and emission wavelength at 590 nm. Higher emission (amount of produced resorufin) is normally proportional to the amount of CYP1A enzyme and the activity in the sample (**Figure 11**).

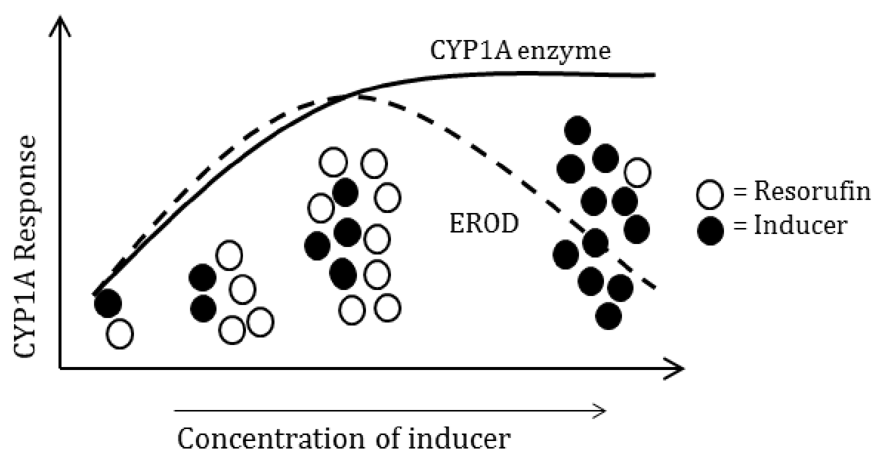

Figure 11. The relationship between inducer, CYP1A enzyme content and EROD activity. Filled dots represent CYP1A enzymes occupied with the inducer and open circles represent free CYP1A enzymes that are available for the ethoxyresorufin substrate and therefore capable of producing the resorufin.

3.3.4 Measurement of 7-benzyloxy-4-(trifluoromethyl)-coumarin-O-debenzyloxylase (BFCOD) activity

In **Paper I**, the CYP3A activity was measured by using 7-benzyloxy-4-(trifluoromethyl)-coumarin as diagnostic substrate (Miller et al., 2000) and optimized for fish liver microsomes (Tove Hegelund Myrbäck, PhD Thesis, 2003). In this fluorescence based assay, termed 7-benzyloxy-4-(trifluoromethyl)-coumarin-*O*-debenzyloxylase (BFCOD) activity, the substrate is catalyzed by the CYP3A enzyme to a fluorescence product 7-hydroxy-4-(trifluoromethyl)-coumarin. The accumulation of the product is

measured in a fluorometer with the excitation wavelength at 405 nm and the emission wavelength at 535 nm.

3.3.5 Quantification of efflux pump activity

In **Paper I**, the efflux pump activity was measured by using rhodamin 123 as a substrate. Rhodamin 123 is a fluorescent dye which diffuses into the cell and is actively pumped out by Pgp and MRPs. Inhibition of the pumps leads to an increased accumulation of rhodamine 123 in the cell. The amount of rhodamin 123 in the cells is measured at excitation/emission wavelengths 485/530 nm using a fluorometer.

3.3.6 CYP1A protein quantification using Western blot

In **Paper III**, the Western blot technique was used to analyze CYP1A protein levels. By using sodium dodecyl sulphate-polyacrylamide gel electrophoresis, proteins can be separated based on size. The proteins are transferred to a nitrocellulose membrane and the target protein can be detected with specific antibodies and enhanced chemoluminescence. Polyclonal rabbit antibodies raised against perch (*Perca fluvatilis*) CYP1A protein, accordingly to Celander and Förlin (1991), were used.

3.3.7 Immunocytochemistry

Immunocytochemistry was used in **Paper I** to stain microtubules. Monoclonal mouse antibodies raised against chicken tubulin (the subunit of microtubules) were used on fixed cultured cells followed by binding of fluorescence-labeled secondary antibodies and visualized by fluorescence microscopy.

3.3.8 Isolation and gene analyzes

In **Paper I**, degenerated reverse transcriptase PCR was carried out in order to isolate partial transcripts of CYP3A from guppy and CYP1A and CYP3A from the PLHC-1 cell line. By using primers designed against conserved regions in the target gene, partial sequence product can be amplified. The product was sequenced by Eurofins MWG operon, Germany and compared to the NCBI database using basic local alignment search tool (BLAST).

In **Paper IV**, a full-length PXR sequence from killifish was cloned. First a partial PXR sequence was obtained by using degenerated primers against conserved regions followed by a 5'- and 3'-RACE in order to get the complete sequence. The PCR product was sequenced by University of Maine DNA Sequence facility, Orono, ME, USA.

3.3.9 Phylogenic analysis

In order to assess the relationship among the vertebrate PXRs ligand-binding domain a phylogenic analysis was carried out as described in **Paper IV**.

4. FINDINGS AND DISCUSSION

This thesis focuses on regulation and function of the AhR-CYP1A signaling pathway and possible interactions with the ER-VTG and PXR-CYP3A/Pgp signaling pathways. Here, relevant toxicological issues are addressed, such as chemical interactions, chemosensitizing and chemical resistance. In addition, this thesis shows that two classical biomarkers are affected by chemical mixture exposure which can lead to under- or overestimation of chemicals in biomonitoring programs.

4.1 Regulation and function of CYP1A (by classical and non-classical AhR ligands)

The CYP1A enzyme has a key role in the metabolism of many environmental pollutants. The induction of CYP1A gene expression is mediated via AhR. Classical CYP1A inducers are PAHs and PHAHs with dioxin being the ultimate AhR agonist. The flavonoid BNF, although it does not belong to either of these classes, acts as a PAH and is traditionally used as a model AhR agonist and CYP1A inducer. Therefore, BNF is used as a positive control for CYP1A induction in **Paper I**, **Paper II** and **Paper III**. Planar and non-*ortho*-substituted PCBs are AhR agonists and potent CYP1A inducers, and the dioxin-like PCB 126 is used as an AhR agonist in **Paper IV**. In addition, a wide range of diverse substances have been shown to bind to AhR and activate AhR mediated responses, such as induction of CYP1A gene expression (Stegeman and Hahn, 1994; Denison et al., 2011). In this thesis, induction of CYP1A was examined in fish and in cultured fish liver cells exposed to a wide range of diverse substances including flavonoids, azoles, PCBs, xenoestrogens and anti-estrogens alone or in mixtures.

4.1.1 Azoles and steroidogenic CYP enzymes

Many azoles are designed and used as antifungal agents as they inhibit the CYP51 activity, which is the key enzyme in the fungal ergosterol biosynthesis (Henry and Sisler, 1984; Vanden Bossche et al., 1995). However, this inhibition is not restricted to fungal CYP51 enzymes and a broad range of CYP enzymes can also be inhibited by azoles in non-target

organisms, including vertebrates. In fish, several azoles have been shown to exert anti-estrogenic effects via inhibition of the aromatase (CYP19) enzyme activity. The CYP19 enzymes catalyze the biosynthesis of estrogen from androgens and inhibition of this activity by azoles can for example cause reduced egg-production or masculinization in female fish (Ankley et al., 2005; Celander et al., 2011). This thesis work focuses on how CYP1A is affected in fish liver cells exposed to different types of azoles.

4.1.2 Azoles and induction of CYP1A

Induction of CYP1A by azoles was studied in **Paper I** and **Paper II,** where it was demonstrate that several imidazoles (i.e. clotrimazole, nocodazole and prochloraz) act as weak CYP1A inducers in PLHC-1 cells. In **Paper I**, exposure to nocodazole and clotrimazole induce CYP1A mRNA levels which is also reflected on the CYP1A (EROD) enzyme activities. In addition, **Paper II** demonstrates that exposure to prochloraz results in increased CYP1A mRNA level and enzyme activity. Induction of CYP1A by fungicide azoles has previously been demonstrated *in vitro* and *in vivo* in rainbow trout (Babìn et al., 2005; Navas et al., 2004; Sturm et al., 2001). The induction of CYP1A by nocodazole, which is a microtubule inhibitor, is surprising as it is questionable as to whether AhR translocation can proceed in cells where assembled microtubules are absent. This is further discussed in section 4.1.5. In addition, in **Paper II**, a trend towards increased AhR mRNA levels is seen by exposure to nocodazole, but no effect of exposure to prochloraz in PLHC-1 cells. Whether azoles mediate CYP1A induction via AhR is not yet clear. Conformational studies indicate that neither clotrimazole nor prochloraz are typical AhR ligands as they are unlikely to adopt a planar structure (Babìn et al., 2005; Navas et al., 2004). However, it has been suggested that the AhR contains a promiscuous binding site to which structural diverse chemicals can bind and activate transcription of target genes (Denison and Nagy, 2003). Furthermore, studies on mammalian hepatoma cell lines show that several azoles have AhR agonistic activities (Long et al., 2003; Korashy et al., 2007; Takeuchi et al., 2008). This supports the hypothesis of an AhR dependent mechanism. The findings in this thesis also indicate that azoles are weak ligands to the AhR. This is supported by the findings that exposures to clotrimazole, nocodazole and prochloraz up-regulated the CYP1A mRNA levels in PLHC-1 cells (**Paper I** and **Paper II**)

and that AhR2 mRNA was elevated by exposure to nocodazole (**Paper II**). Earlier studies in our lab show that the imidazole ketoconazole induces CYP1A mRNA and enzyme activities in fish *in vivo* (Hegelund et al., 2004; Hasselberg et al., 2005; 2008). Recently, exposure to prochloraz either via water (150 mg L^{-1}) or intra peritoneal injections (50 mg kg^{-1} fish) resulted in induced CYP1A mRNA levels in sand gobies (*Pomatoschistus minutus*) of both sexes (Gräns et al., unpublished data). Still, it cannot be determined by which mechanism CYP1A is induced by azoles in fish. There might be an azole metabolite which is activating the AhR rather than the parent compound, or there may be an alternative intracellular signaling mechanism. In human hepatocytes, CYP3A metabolism has been suggested to be involved in the induction of CYP1A by an omeprazole metabolite by conversion to an AhR ligand (Gerbal-Chaloin et al., 2006). However, PLHC-1 cells seem to lack a functional PXR-CYP3A signaling pathway, which indicates that CYP3A-activation of azoles does not occur in this cell line (**Paper I**). Yet, it cannot be ruled out that other enzymes are involved in CYP1A induction in PLHC-1 cells upon exposure to azoles. Nevertheless, significant induction of CYP1A in PLHC-1 cells exposed to nocodazole was evident as early as 6 hours after exposure (**Paper II**). This implies that azoles have a direct effect on AhR signaling rather than a metabolite binding to AhR. This could be a possible defense mechanism for at least partly overcome the inhibition of the CYP1A enzyme by azoles.

4.1.3 Azoles and inhibition of CYP1A

In addition, functional studies on CYP1A enzyme activity were investigated by exposure to azoles in **Paper I** and **II**. In **Paper I,** clotrimazole, nocodazole and ketoconazole act as CYP1A inhibitors in PLHC-1 cells, in descending order (**Table 2**). The inhibition of hepatic CYP1A by clotrimazole and ketoconazole is consistent with other studies of imidazoles in fish (Levine et al., 1997; Hegelund et al., 2004; Hasselberg et al., 2005; Lennquist et al., 2008; Burkina et al., 2013). Ketoconazole was shown to be a potent inhibitor of CYP1A activity in hepatic microsomes with IC_{50} concentrations at 0.6 µM and 0.4 µM, from BNF treated juvenile Atlantic cod (*Gadus morhua*) and juvenile rainbow trout, respectively (Hegelund et al., 2004; Hasselberg et al., 2005). In addition, clotrimazole acted as a potent CYP1A enzyme activity inhibitor in hepatic microsomes,

with IC_{50} concentrations at 0.5 μM 0.6 μM, from gizzard shad and rainbow trout respectively (Levine et al., 1997; Burkina et al., 2103). Furthermore, in **Paper II**, the antifungal agent prochloraz is also shown to act as a CYP1A enzyme activity inhibitor in PLHC-1 cells and in BNF treated juvenile rainbow trout microsomes (**Table 2**). When comparing the studies, the IC_{50} concentrations are higher in PLHC-1 cells, that is, higher concentrations of the azoles are needed to inhibit 50 percent of the CYP1A enzyme activity. The variance is most likely a model system difference. In the microsomes, inhibition is measured directly in the S9-fraction, while in the PLHC-1 cell line the inhibition is measured in intact cells in which cellular processes, as uptake of the inhibitor, influence. The kinetics of CYP1A inhibition was further investigated in rainbow trout liver microsomes to characterize the type of inhibition exerted by prochloraz. The current results suggest that prochloraz acts as a mixed-type inhibitor of CYP1A. Previous studies of imidazoles in fish demonstrate non-competitive or mixed inhibition in the sub-μM range (Snegaroff and Bach 1989; Levine and Oris, 1999; Hasselberg et al., 2005; Burkina et al., 2013). A non-competitive inhibitor and a mixed inhibitor can both bind to an allosteric site on the enzyme, regardless of whether a substrate has already bound or not to the active site. (Ring et al., 2014). Consequently, inhibition of CYP1A can lead to impaired ability to metabolize xenobiotics which can result in an increased sensitivity to chemical exposure as well as accumulation of xenobiotics to toxic concentrations. What this mean for individuals, populations and ecosystem is difficult to predict and further studies are needed. However, disrupted reproduction, developmental effects, tumors and mortality are possible adverse effects for exposed fish. In addition, inhibition of CYP1A enzyme activity by azoles can also result in misinterpretation of biomarker data by underestimation of aromatic hydrocarbons, and thus give a wrong picture of the environment status.

Table 2. Median inhibition (IC_{50}) concentrations for azoles on CYP1A activity *in vitro*.

	IC_{50} value ± SE (μM)	
Substance	**PLHC-1 cells**	**Microsomes**
Clotrimazole	1.3± 0.5	Not analyzed
Ketoconazole	4.0± 1.6	Not analyzed
Nocodazole	1.7± 0.8	Not analyzed
Prochloraz	7.7± 1.4	0.07 ± 0.02

4.1.4 EDCs and effects on CYP1A

Exposure to EE_2 and bisphenol A, which both are ER agonists, has no effect on CYP1A activities in PLHC-1 cells, and bisphenol A has no effect on CYP1A mRNA levels (**Paper I**). In contrast, primary cultures of rainbow trout hepatocytes exhibited down-regulated basal levels of CYP1A mRNA and CYP1A activities by exposure to EE_2 (**Paper III**). In an earlier study, brook trout (*Salvenius fontinalis*) exposed to E_2 *in vivo* had reduced amount of total CYP protein levels (Pajor et al., 1990). Lower levels of CYP1A proteins and catalytic activities in females compared with males have been reported in fish (Elskus et al., 1989; Arukwe and Goksøyr, 1997). In Atlantic cod, in contrast, females had higher CYP1A protein levels than males and exposure to E_2 or estrogenic alkylphenols *in vivo* resulted in induction of CYP1A protein levels (Hasselberg et al., 2004). Hence, estrogens and estrogenic EDCs can interfere with CYP1A expression. Other EDCs that are anti-estrogenic can also interfere with CYP1A. For example, exposure of PLHC-1 cells to the anti-estrogenic and mammalian ER-antagonist fulvestrant for 24 hours up-regulates CYP1A mRNA levels, but not CYP1A enzyme activities. In addition, acute exposure (10 minutes) to fulvestrant has no effect on CYP1A activity in this cell-line (**Paper I**). The same pattern is seen in primary cultures of rainbow trout hepatocytes where exposure to fulvestrant for 24 hours up-regulates CYP1A mRNA levels, but not CYP1A activities (**Paper**

III). However, inhibition studies in rainbow trout liver microsomes show that fulvestrant acts as a potent inhibitor of the CYP1A activity (**Paper III**). This thesis shows that xenoestrogens and anti-estrogens interact with CYP1A either on mRNA levels and/or on catalytic function. For fish exposed to xenoestrogens and anti-estrogens in their natural habitat, this can result in reduced metabolism and thereby increase the sensitivity to exposure of these substances. EDCs affecting the estrogen signaling system can lead to adverse effects and endocrine disruption. Endocrine disruption, including feminization and masculinization, has been evident in fish near sewage treatment plants which can have negative effects on reproduction.

4.1.5 Microtubules and CYP1A induction

Induction of CYP1A is a dynamic process that is regulated by AhR signaling, which includes ligand activation in the cytoplasm, translocation to the cell nucleus and binding to AhR response element in the CYP1A gene (Schmidt and Bradfield, 1996; Denison et al., 2011; **Figure 6**). An intact cytoskeleton has been shown to be important for AhR-CYP1A signaling in mammalian hepatic cells (Dvořák et al., 2006). Microtubules are essential for energy-dependent cellular transport (Dumontet and Jordan, 2010). Nocodazole is an anti-neoplastic agent which acts by disassembly of the microtubules thereby removing the tracks for active cellular transport. Studies on mammalian cell lines and hepatocytes show a decreased AhR ligand mediated induction of CYP1A mRNA in cells pre-treated with nocodazole (Dvořák et al., 2006; Schöller et al., 1994; Vrzal et al., 2008). However, in contrast to those studies, a significant induction of CYP1A mRNA levels is seen in PLHC-1 cells exposed to nocodazole. This was unexpected since disassembly of microtubule was evident in these cells (**Paper I**).
The phenomenon can be explained by the following hypotheses:
1) Microtubules are not involved in AhR translocation.
2) The AhR is already located in the nucleus in this cell line.
3) Microtubule disassembly is not instant and translocation of AhR occurs before disassembly of the microtubules.
4) Nocodazole induces CYP1A by an AhR independent mechanism.

The role of microtubules in AhR-CYP1A signaling was further investigated in **Paper II**, where PLHC-1 cells were pre-treated with nocodazole for

either 5- or 24 hours, followed by exposure to the AhR ligand BNF for an additional 24 hours. Pre-treatment with nocodazole has no effect on the BNF mediated induction of CYP1A, as measured by CYP1A activity, compared with cells pre-treated with the solvent followed by 24 hours exposure to BNF (**Paper II**). The results therefore show that induction of CYP1A enzyme activity is not affected by a lack of assembled microtubules. However, the highest dose of nocodazole, pre-exposed for 5 hours, resulted in a slightly decreased induction of CYP1A enzyme activity compared to cells exposed to BNF alone. However this decrease is most likely due to inhibition at the enzyme level rather than a result of disrupted transport of AhR. Consequently, AhR translocation is probably not microtubule-dependent in this cell line, even if hypothesis that AhR is already located in the nucleus cannot yet be ruled out. In order to find out whether this is the case, immunocytochemistry and commercially available polyclonal antibodies raised in goat against a human AhR epitope, recommended for detection of AhR in zebrafish were used. However, these antibodies fails to recognize AhR in PLHC-1 cells and this question still remains open (**Paper II**). Another alternative is that the AhR is translocated to the nucleus by passive diffusion. The glucocorticoid receptor signaling has been suggested to proceed via microtubule dependent translocation under normal conditions in a mouse fibroblast cell line, but when microtubules are disassembled, the glucocorticoid receptor moves toward the nucleus by diffusion (Galigniana et al., 1998). This might also be the case for AhR translocation in the PLHC-1 cell line. Further studies are needed to clarify the AhR transport mechanism. A more thorough search and optimization of an antibody which recognizes AhR in PLHC-1 is desirable in order to find its cellular localization and to perform AhR transport studies. However, the translocation of AhR might be a rather complex system involving several mechanisms, where one does not need to exclude another.

4.2 Function and regulation of CYP3 in the PLHC-1 cell line

4.2.1 Identification of a PLHC-1 CYP3 gene

The PLHC-1 cell line has become a commonly used hepatic model in fish studies of toxicity and metabolism as it has been shown to have several detoxification system components. In vertebrates, including fish, CYP3A is the dominant hepatic CYP form and responsible for metabolisms of a wide range of structurally diverse chemicals (Celander et al., 1996a; Hegelund and Celander, 2003). However, there is a lack of information about CYP3A in the PLHC-1 cell line, even if a previous study showed the presence of a putative CYP3A protein in PLHC-1 cells (Celander et al., 1996b). Besides, CYP3A-like (i.e. BFCOD) activities have been reported in PLHC-1 cells (Christen et al., 2009). By using a suite of degenerate PCR primers targeted for fish CYP3A cDNAs, a thorough search for a CYP3A orthologue in PLHC-1 cells was carried out without success (Maria Fernandéz, MS Thesis, 2011). In order to obtain more species-specific PCR primers, a partial CYP3A sequence was cloned from a closely related species, the guppy (*Poecilia reticulata*). This sequence was used to design new gene specific primers for guppy CYP3A, and a 216 base pair long cDNA sequence was isolated from the PLHC-1 cell line. By using the NCBI protein BLAST, the deduced amino acid sequence was compared to other proteins. The PLHC-1 sequence shared 69% sequence identity with the CYP3A40-like protein from Nile tilapia (*Orechromis niloticus*), 60% with CYP3C1 and 58% with CYP3A65 from zebrafish. This raises the question of to which subfamily within the CYP3 gene family the obtained PLHC-1 sequence actually belongs. The obtained sequence was tentatively referred to as CYP3A in **Paper I**. As it is possible that this gene belongs to another CYP3 subfamily, phylogenetic analysis was subsequently carried out by Dr. Joanna Wilson. The phylogenetic tree revealed that the obtained sequence most likely is a member of the CYP3B subfamily (Britt Wassmur, PhD Thesis 2012). Therefore, the sequence is called CYP3B in this thesis. However, the question of the presence of a CYP3A gene in PLHC-1 cells still remains, but appears more unlikely after this thorough search. Regardless, measurement of CYP3 induction and enzyme activity may not be ideal in this cell line. Instead, this cell line can be used to study other processes without the

interference of CYP3A which can be advantageous when studying CYP1A enzyme activities.

4.2.2 Regulation and function of CYP3B

To further investigate responsiveness of CYP3B in PLHC-1, induction and activity were measured in **Paper I**, following exposure to a wide range of structurally different substances including several PXR agonists which are known prototypical mammalian CYP3A inducers. Here, none of the investigated substances induced the CYP3B mRNA levels in PLHC-1 after 24 hours exposure. The result are consistent with previous studies where exposures to mammalian PXR agonists had no effect on CYP3A-like protein levels in PLHC-1 cells (Celander et al., 1996b) or on CYP3A mRNA levels in the rainbow trout hepatoma (RTH-149) cell line (Wassmur et al., 2010). In addition, low CYP3A mRNA basal levels were seen in RTH-149 (Wassmur et al., 2010), as well as in human hepatoma (HepG2 and Mz-Hep1) cell lines (Rodríguez-Antona et al., 2002). A low basal CYP3B mRNA level was apparent in PLHC-1 cells and, in fact, it was lower than that of CYP1A in untreated cells (**Paper I**). In addition, the CYP3A-like enzyme activity is not altered by exposure to mammalian PXR agonist such as pregnenolone-16α-carbonitrile, rifampicin and other pharmaceuticals (**Paper I**) which are known to induce CYP3A activity in mammals. Previous data indicate that CYP3A-like activity is low in the PLHC-1 cell line (Thiabut et al., 2009). However, another study reports induced CYP3A-like (BFCOD) activities by exposure to the PXR agonist rifampicin (Christen et al., 2009). There are no CYP3A specific diagnostic substances and hence several CYP isoforms, including CYP1A, can also catalyze the BFCOD activity (Price et al., 2000). This was not addressed by Christen et al., (2009). It is possible that the reported induction of BFCOD activity in PLHC-1 cells upon rifampicin exposure is catalyzed by another enzyme rather than CYP3A or CYP3B. In conclusion, the low responsiveness of CYP3A in cell lines is confirmed by the thesis results. The reason for lack of CYP3A/CYP3B induction and enzyme activity in the PLHC-1 cell line remains unclear. In RTH-149, the basal level of PXR is much lower than in rainbow trout hepatocytes (Britt Wassmur, PhD Thesis 2012). Hence, one explanation can be that this cell line has lost or has a less functional PXR and thereby has impaired ability to metabolize CYP3A/CYP3B substrate. The PLHC-1 cell line, when studying

CYP1A and efflux regulation in screening studies, is still of value. However, one must be aware of the deficiencies and take these into account when interpreting the data. Additionally, results should be validated by using another approach, such as primary cultures or *in vivo* experiments.

4.3 Function and regulation of efflux pumps

Other important key players in the detoxification and defense against xenobiotics are the efflux pumps. **Paper I** explores how structurally diverse substances interact with the function and regulation of toxicological relevant efflux pumps, such as MRPs and Pgp.

4.3.1 Efflux inhibition

Efflux activities were screened for 18 different substances by using the rhodamine 123 bioaccumulation assay. Of these 18 substances, the non-steroidal anti-inflammatory drug (NSAID) diclofenac and the macrolide antibiotic troleandomycin inhibited efflux activities in PLCH-1 cells (**Figure 12**; **Paper I**). Efflux inhibitors can be described as chemosensitizers because they can increase accumulation and thereby toxicities of other compounds as a consequence of reduced efflux capacities (Smital and Kurelec, 1998). The inhibition of efflux activity most likely results from competition with rhodamine for the active site on the efflux pump. At this stage it is not possible to know which pump or pumps that are affected as rhodamine can be transported by both Pgp and MRPs (Kim, 2002). Exposure to efflux inhibitors, can in situations of mixture exposure, lead to increased accumulation of other xenobiotics to harmful concentrations. Accumulation of xenobiotics may cause multiple adverse toxic effects for individual fish and further studies are needed to evaluate how that may affect the fitness of a fish and population.

4.3.2 Efflux activation

In addition to inhibition of efflux activities, certain substances can act as activators and thereby enhance efflux. In the thesis studies, it is demonstrated that EE_2 directly activates efflux pump activity by decreasing rhodamine accumulation (**Figure 12**; **Paper I**). It has earlier been proposed

that Pgp contains an additional third allosteric site to which prazosin and progesterone can bind, without being substrates themselves, and stimulate transports from other drug-binding sites (Shapiro et al., 1999). The thesis results support the hypothesis of a third allosteric site to which EE_2 can bind and stimulate efflux. This type of direct stimulation of efflux activity has previously been demonstrated in a human trophoblast-like (BeWo) cell line following exposure to bisphenol A, and it was suggested that the third binding site could explain this efflux activation (Jin and Andus, 2005). Here, exposure to bisphenol A does not significantly affect the efflux activity, even though a slightly enhanced efflux activity is seen (**Figure 12**; **Paper I**). Interestingly, both EE_2 and bisphenol A appear to activate efflux activity and they are both ER-agonists. Future investigations should address whether ER signaling interacts with efflux activity. Increased efflux activities can potential lead to an accelerated efflux of e.g. endogenous substances and lead to depletion of for example hormones which could lead to endocrine disruption

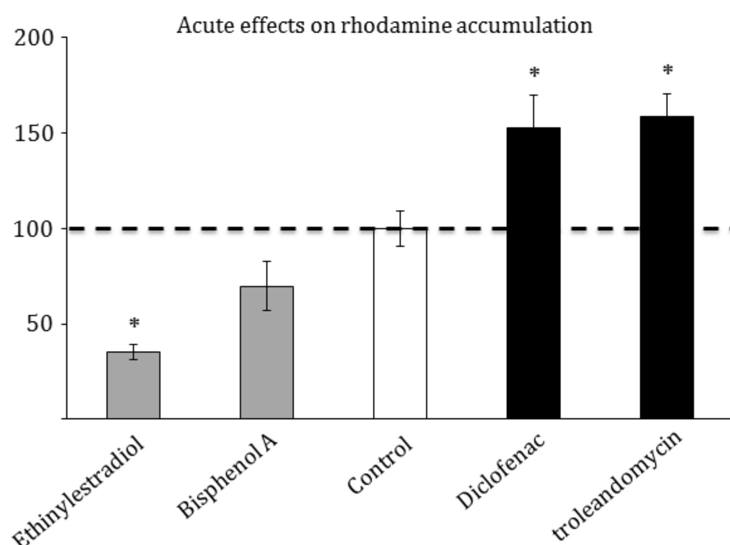

Figure 12. Rhodamine bioaccumulation in PLHC-1 cells. The figure is modified from **Paper I.**

4.3.3 Efflux induction

In addition to acting as an efflux inhibitor, troleandomycin also induces the MRP2 mRNA level which might be a response that can prevent accumulation. Hence, it is possible that induction of MRP2 mRNA is a

response to efflux inhibition in order to restore the efflux capacity in these cells. The MRP2 mRNA level was also up-regulated after exposure to BNF (**Paper I**). This is in accordance with recent studies, demonstrating that MRPs are being induced by AhR agonists *in vitro* and *in vivo* in fish (Costa et al., 2012; Ferreira et al., 2014; Yuan et al., 2014). However, BNF had no direct effect on the efflux activity and is probably not a substrate to the efflux pumps (**Paper I**). It has been suggested from a study, using a mouse reporter assay, that BNF up-regulates MRP2 via the antioxidant response element and nuclear factor-E2 related factor 2 (Vollrath et al., 2006). In addition, a coordinated regulation of MRPs by several receptors including AhR and PXR has been demonstrated in mice (Aleksunes and Klaassen, 2012; Maher et al., 2005). Hence, it is possible that different signaling mechanisms are inducing MRP2 by exposure to BNF compared to troleandomycin.

4.4 Mixture effects

Although most chemicals occur as mixtures in the environment, chemical risk assessments are mainly based on single chemical tests (Backhaus and Faust, 2012; Kienzler et al., 2014). In those legislations taking mixtures into account, mathematical models are used to assess the combined toxicological effects. The most commonly model used are the concentration-addition model and the independent action model which both are based on the assumption that there is no interaction between substances. The concentration-addition model is applied to chemical mixtures which share the same mode of action and predicts an additive response. Independent action model is used when a chemical mixture affect the same end point although with different mode of actions (Backhaus and Faust, 2012; Kortenkamp, 2014). The two additive models that are based on linear effects may not always be applicable, in particular in situations with non-linear synergistic or antagonistic mixture effects. This can be in part due to the fact that chemicals with either the same or different modes of action may share common elimination pathways in phase 0-3 (Celander, 2011; Cedergreen, 2014; **Figure 7**). This can alter the biochemical half-life of different chemicals. The studies of this thesis show that many diverse chemicals interact with CYP1A and efflux in fish hepatic cells (**Paper I**,

Paper II and **Paper III**). These interactions can occur either at the receptor level which affects regulation of target genes, or directly on the function of the enzyme or transporter protein (Celander, 2011). Interactions between different chemicals at receptor or enzyme levels can result in synergistic or antagonistic responses. Antagonism occurs if the sum of response in the mixture is lower than the sum of the response by the individual chemicals. In contrast, synergism entails that the sum of response in the mixture is higher than the sum of the individual chemicals. This can be calculated using the following equation:

$$\sum CYP1A_{mixture} - (CYP1A_{chemical\ A} + CYP1A_{chemical\ B}) \neq 0$$

4.4.1 Synergistic effects on CYP1A after co-exposure to azoles and BNF

Synergistic effects on CYP1A in fish by exposure to a PAH mixture, including a strong inducer and a weak inducer, has previously been reported (Basu et al., 2000; Timme-Laragy et al., 2007; Gunawickrama et al., 2008). The effects on CYP1A induction and activity after combined exposure to BNF with either of three different azoles were studied in **Paper II**. Exposure to all examined imidazoles, prochloraz, clotrimazole and nocodazole, for 24 hours cause synergistic increasing CYP1A enzyme activities, 16-fold at the most with nocodazole (**Table 3**). For nocodazole this is also reflected on CYP1A mRNA levels, where up to a 40-fold synergistic increase occurs (**Table 3**). No mixture effect on AhR2 mRNA levels is seen after 24 hours exposure. However, the synergistic effect of the prochloraz-BNF mixture on CYP1A enzyme activities after 24 hours exposure is not reflected in CYP1A mRNA levels (**Table 3**) or AhR2 mRNA levels. As azoles act as strong inhibitors (**Paper I**, **Paper II**, Levine et al., 1997; Hegelund et al., 2004; Hasselberg et al., 2005; Lennquist et al., 2008; Burkina et al., 2013), the synergistic effect seen by co-exposure of azoles and BNF is most likely a result of inhibition on CYP biotransformation and thus preventing elimination of BNF which prolongs and enhance CYP1A induction. The reason why the synergistic effect is only reflected in CYP1A mRNA levels for nocodazole and not for prochloraz is not clear. A possible explanation is that nocodazole is a stronger inhibitor than prochloraz (**Table 2**; **Paper I**; **Paper II**) and thus more BNF remains for a longer time in the cells exposed to nocodazole. Induction of mRNA levels is a relatively fast response and

thus after 24 hours, the amount of CYP1A mRNA has already decreased in the cells co-exposed to prochloraz. Levels of mRNA could be measured earlier, 6 or 12 hours after exposure to determine if CYP1A mRNA levels are synergistic induced. To further elucidate the synergistic effect, time-course experiments were conducted measuring CYP1A activities 6, 12, 24, 48 and 72 hours after exposure to three doses of nocodazole in combination with either 0.1 µM BNF or 1 µM BNF. The presence of nocodazole increases the sensitivity to BNF up to one order of magnitude and prolongs the response to BNF treatment by 24 hours. Fish exposed to azoles in their natural habitat can become more sensitive for exposure to chemicals that are metabolized by CYP1A enzymes and lead to accumulation to harmful concentrations. An enhanced and prolonged CYP metabolism can lead to increased production of reactive oxygen species and toxic metabolites. Consequently, this can lead to toxicity by the mixture that is not seen by single exposures.

Table 3. Synergistic effects on CYP1A enzyme activity and CYP1A mRNA levels in PLHC-1 cells after combined exposure to BNF with either of the azoles. The synergy fold is calculated by using following equation $\sum CYP1A_{mixture} / (CYP1A_{chemical\ A} + CYP1A_{chemical\ B})$ ±SE

	CYP1A synergy (fold)					
	Activity			mRNA		
Substances	1 µM	10 µM	25 µM	1 µM	10 µM	25 µM
Clotrimazole	3±0.4	1±0.2	Not analyzed	Not analyzed	Not analyzed	Not analyzed
Nocodazole	2±0.7	9±1.8	16±3.5	0.4±0.05	3±0.7	40±9
Prochloraz	4±0.6	3±0.5	Not analyzed	0.2±0.02	0.4±0.05	Not analyzed

4.4.2 AhR-ER cross-talk in fish

The interaction between AhR and ER was investigated in **Paper III**. Primary cultures of rainbow trout hepatocytes were exposed to the AhR agonist BNF and ER agonist EE_2 alone and in combinations. To evaluate possible cross-

talk, mRNA levels for AhR, CYP1A, ER and VTG as well as CYP1A enzyme activities and CYP1A protein levels were determined. Here, there appears to be a one-directional inhibition of AhR-ER cross-talk. That is, exposure to BNF has an anti-estrogenic effect by decreasing the EE_2-mediated induction of ER and VTG mRNA levels. Exposure to EE_2, on the other hand, has no effect on the BNF mediated responses on AhR and CYP1A mRNA levels or on CYP1A protein levels. However, the CYP1A enzyme activity is decreased in cells exposed to BNF and EE_2 compared to cells exposed to BNF alone. It has been shown that EE_2 can inhibit the CYP1A mediated EROD activity *in vitro* in fish liver microsomes (**Paper I**; **Paper III**; Hasselberg et al., 2005). As neither CYP1A mRNA nor protein levels were affected by EE_2 exposure of rainbow trout hepatocytes, the observed reduction of CYP1A activities is likely a result of inhibition of CYP1A enzyme level rather than on AhR receptor level (**Paper III**).

There are previous studies addressing interactions between AhR and ER in fish or fish liver cells exposed to different AhR ligands and/or ER ligands. For example, decreased VTG protein levels have been reported in rainbow trout hepatocytes exposed *in vitro* to BNF or the PAH 3-methylcholanthrene in combination with E_2 (Navas and Segner, 2000). Furthermore, in rainbow trout exposed *in vivo* to BNF and E_2, plasma VTG levels decreased (Anderson et al., 1996). In addition decreased levels of VTG mRNA and ER mRNA occur in Atlantic salmon hepatocytes co-exposed *in vitro* to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and E_2 (Bemanian et al., 2004). Accordingly, these studies demonstrate a one-way inhibitory cross-talk between AhR and ER. In contrast, reciprocal cross-talk has been demonstrated in Atlantic salmon (*Salmo salar*) and rainbow trout hepatocytes. As the nonylphenol induced ER- and VTG mRNA levels are decreased after co-exposure with PCB 77. In addition, the PCB 77 induced AhR- and CYP1A mRNA levels are decreased after co-exposure with the estrogenic nonylphenol (Mortensen et al., 2006; Mortensen and Arukwe, 2007).

The reasons for these contrasting results are not clear. There are likely several factors, such as species differences, receptor agonists, timing and methods, which can influence the receptor interaction. This illustrates the complexity of receptor interactions which all have to be taken into account for e.g. biomonitoring programs.

4.4.3 Mixture effects on biomarker responses

The thesis data show that two commonly used biomarkers are affected during mixed exposure. Induction of CYP1A is commonly used to assess the occurrence of aromatic hydrocarbons in the aquatic environment. In **Paper II**, synergistic interactions between the PAH model compound BNF and azoles are found, resulting in increased CYP1A enzyme activity. This can lead to an overestimation of levels of aromatic hydrocarbons in the aquatic environment when azoles also are present. Furthermore, in **Paper III** co-exposure to BNF with EE_2 resulted in decreased CYP1A enzyme activity. This can lead to underestimation of aromatic hydrocarbons in the aquatic environment when estrogenic compounds are present. In addition, to assess estrogenic compounds in the aquatic environment induction of VTG is frequently used. In **Paper III**, an inhibitory cross-talk between AhR and ER is observed, resulting in a decreased response of the VTG mRNA expression. This can lead to an underestimation of levels of estrogenic compounds in the aquatic environment when AhR ligands also are present. These data strongly support the urgent need for the development of new approaches to evaluate effects of mixture exposure both in aquatic biomonitoring program and in chemical risk assessments.

4.5 Chemical resistance

New Bedford Harbor (NBH) is heavily contaminated with PCBs. Killifish in NBH can survive and reproduce, despite the presence of high levels of PCBs, by reducing the AhR-CYP1A signaling pathway (Nacci et al., 1999; Bello et al., 2001). In **Paper IV**, the expressions of PXR, CYP3A and Pgp genes are investigated in fish from NBH and compared with killifish from the nearby Scorton Creek (SC) reference site. In addition, responses to PCB 126 and PCB 153 exposure in the laboratory were investigated. Killifish from NBH respond to both the dioxin-like PCB 126 (AhR agonist) and the non-dioxin like PCB 153 (mammalian PXR agonist) exposures with increasing hepatic PXR, CYP3A and Pgp mRNA levels, which is not seen in fish from SC (**Paper IV**). The reason for the differences between the populations is not clear. The inducibility of CYP3A has previously been correlated to lower basal levels of PXR and CYP3A in zebrafish larvae and rainbow trout hepatocytes (Tseng et

al., 2005; Wassmur et al., 2010). The basal mRNA levels of PXR and Pgp are lower in fish from NBH than in fish from SC, which could be an explanation to the differences, but further studies are needed.

The data confirm a reduced AhR-CYP1A signaling response in NBH killifish by exposure to PCBs. In contrast, these fish respond to PCBs by induction of hepatic PXR, CYP3A and Pgp RNA levels. The mechanism for this reduced AhR-CYP1A response is not yet clear although very intriguing. The results indicate a possible involvement of PXR, CYP3A and Pgp in PCB resistance. The following section 4.5.1 addresses a possible inhibitory PXR-AhR cross-talk mechanism in NBH fish different from that in SC fish.

4.5.1 Chemical resistance mechanisms: Putative receptor cross-talk?

In mammals, cross-talk between PXR and AhR has been reported. In human hepatocytes, AhR and its target genes CYP1A1 and CYP1A2 were regulated by PXR (Maglich et al., 2002). In human cell lines, an AhR agonist induced CYP3A4 through activation of PXR (Kumagai et al., 2012). In fish, induction of CYP3A by AhR agonists has also been reported. Induction of CYP3A65 by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin has been reported in zebrafish larvae (Tseng et al., 2005). In rainbow trout hepatocytes, BNF exposure resulted in a slight induction of CYP3A27 mRNA levels (Gräns and Celander, unpublished). The study of this thesis show that both PCB congeners, PCB 126 and PCB 153, up-regulates the hepatic PXR, CYP3A and Pgp mRNA levels in NBH fish. The opposite is shown in SC fish with no effect on PXR-CYP3A/Pgp signaling by any of these congeners and a powerful induction of AhR-CYP1A signaling upon treatment with PCB 126, and weaker induction of CYP1A with PCB 153. The fact that a weak induction of AhR-CYP1A signaling is obtained in fish from NBH implies that AhR is still activated but less functional. This suggests the possibility of cross-regulatory interactions between PXR and AhR signaling pathways in this population where PXR suppresses AhR activation in NBH fish.

The toxicity of non-*ortho* PCBs is believed to primarily be mediated via the AhR, so here reducing the AhR signaling by a putative cross-talk is beneficial for NBH fish. However, further studies are needed to elucidate the possible cross-talk and mechanism in NBH killifish.

5. Conclusions and future perspectives

Although chemical end up as mixture in the aquatic environment, chemical risk assessments are based on single chemical exposure experiments. Chemical mixtures can cause chemical interactions resulting in adverse responses not seen by exposure to chemical alone. The knowledge how fish are affected by chemical mixtures is deficient. In this thesis, effects on the detoxification pathway and biomarker responses in fish exposed to chemicals alone and in mixtures are addressed. The results in this thesis provide increased knowledge of chemical mixture and reveals possible sites for chemical interactions that is important for taking the understanding of mixture toxicity forward.

The studies in this thesis show various types of chemical mixture effects. Here azoles, which are potent CYP1A inhibitors, in combination with BNF cause a synergistic effect on CYP1A induction. This can lead to misinterpretation of biomarker data when AhR ligands together with azoles are present in the aquatic environment. The synergistic effects seen by co-exposure of azoles and BNF are most likely a result of inhibition on CYP1A biotransformation that results in reduced elimination capacity of BNF. In addition, the induction of CYP1A by BNF was prolonged and enhanced in combination with azoles. Potential risks with an increased CYP1A induction can be increased production of reactive oxygen species and other AhR-CYP1A mediated toxic effects. It would therefore be interesting to conduct an experiment addressing possible adverse effects, after co-exposure with an azole and an AhR ligand which themselves has no effect. Furthermore, a one-way inhibitory AhR-ER cross-talk is shown in primary cultures from rainbow trout. This may result in misinterpretation of biomarker data, leading to that the levels of estrogenic compounds in the aquatic environment are underestimated.

Pre-exposures to the microtubule disrupter nocodazole has no effect on the ability of BNF induce CYP1A. This suggests that induction of CYP1A in the PLHC-1 cell line not is microtubule dependent. The antibody used here failed to recognize AhR in PLHC-1 so the question whether AhR is already located in the nucleus is still open. A more thorough search and after an

antibody recognizing AhR in PLHC-1 is desirable to find the cellular localization of AhR and the transport mechanism.

The PLHC-1 cell line is a common ecotoxicology *in vitro* model and suitable for CYP1A and efflux pump studies. So far, a CYP3A gene has not been found in PLHC-1. Here, a CYP3A-like gene was sequenced and a subsequent phylogenetic analysis revealed that it belongs to CYP3B subfamily. In addition, this CYP3B in PLHC-1 was not inducible by exposure to different substances including mammalian PXR agonist. This demonstrates that studying CYP3A/CYP3B responses in PLHC-1 is not suitable.

Killifish from NBH can, by have reduced AhR-CYP1A signaling, survive high levels of PCBs. How PXR- and CYP3A- mRNA levels are affected in NBH killifish is not yet known. Results in this thesis show that the basal level of hepatic PXR mRNA is lower in NBH compared to the reference site SC. In addition, fish from NBH responded to PCB treatment with increasing hepatic PXR, CYP3A and Pgp mRNA levels. Here a putative AhR-PXR cross-talk is suggested. However, further studies are needed in order to confirm this.

Acknowledgements

Till alla som frågar mig hur det är att doktorera svarar jag alltid att det är som den bästa godispåse man kan tänka sig, med många gobitar i. Detta är till stor del tack vara alla er som jag har träffat på vägen och på olika sätt hjälpt mig att nå hit.

Framförallt vill jag tacka:

Malin Celander, min handledare, som stöttat, guidat och trott på mig hela vägen. Du har gett mig utrymme att tänka själv, få komma med idéer och utvecklas. Tack för allt!

Margareta Wallin, min biträdande handledare som alltid hjälper till snabbare än blixten när det behövs.

Thrandur Björnsson, min examinator, för goda råd och uppmuntran.

Britt Wassmur, min bästa Piff och CYP-vän. Hur skulle detta gått utan dig och ditt förnuft, jag vill inte ens tänka tanken. Jag är så glad över att jag fått lära känna dig.

Linda Hasselberg Frank, som alltid har nära till skratt och får mig att orka det där lilla extra. Tack för behövliga kaffepauser och härliga springturer.

Elisabeth Norström, för goda råd och hjälp gällande det mesta. Jag är så glad över att du bestämde dig för att vara kvar på Zoologen min tid ut.

Joanna Wilson, for nice chats and laughter with you and your lovely family, Andrew, Galen and Raynham. Look forward to much collaboration in the future.

Nancy Denslow, for making it possible for me to come and visit your lab in Florida, which was a fantastic experience. Thank you for your hospitality.

John Stegeman, for nice collaboration with the killifish paper.

Bethanie Carney Almroth, särskilt för att du tog dig tid att läsa igenom kappa.

Ola Svensson, för bilturer Sverige runt och som tålmodigt lärt mig allt jag kan om beteendet hos sandstubb.

Till alla på Zoologen, vad ni är bra. Speciellt tack till, *Ål-gruppen* som med öppna armar låtit mig bli en del av gänget. *Ann-Sofie Olsson* och *Lena Sjöblom* som alltid tar sig tid att svara på alla dumma frågor angående de administrativa, oavsett hur mycket ni än har att göra. *Bernt Carlsson* och *Lilioth Olsson* för att ni håller hela huset flytande. Nuvarande och tidigare doktorander, speciellt tack till: *Marcus* för trevligt helgsällskap och tekniksupport. *Jeroen*, for always being the festfixare. *Eva-Lotta,* för avslappnat häng

på Kristineberg. *Lars Niklasson* och *Andreas Kullgren* för sköna springturer*. Albin*, det är gott att du fortfarande är kvar och kan representera Gränsklanen.

Former students at CYP-lab, especially *Marie Michelová* and *Junko Johansson,* for really good work with Paper IV.

Till alla er utanför Zoologen

Susann Werner, det är till stor del tack vara dig jag sitter här idag. Det var du som peppade och fick mig att börja plugga.

Mina gamla pluggkompisar, *Emma Gyllbäck* som i Stockholm fick mig att flitigt sitta och plugga citronsyracykeln. *Sara Calabrese* som gjorde studentlivet i Göteborg så mycket roligare.

Mina fina vänner, *Therese, Märta och Rosie*, även fast vi inte ses så ofta vet jag att ni alltid ställer upp. *Lina Uhac*, för att du alltid säger vad du tycker, inga konstigheter. Jag är glad över att jag fått vara din vän i hmm…..28 år och många år framöver hoppas jag.

Jesper Halling, för den otroligt snygga bilden på framsidan

Kennet Axberg, för att du alltid har funnits till hands, jag är skyldig dig så sjukt mycket.

B*rorsnor*, den finaste bror som kan tänkas.

Min *mamma* och *pappa*, som alltid har stöttat och ställt upp oavsett vad det än gäller. Det finns nog inget ni inte tror jag klarar. Tack för allt!

Maja, för att Du är Du, fantastisk på alla sätt! Tack för ditt oändliga stöd och peppande under skrivprocessen, det skulle aldrig ha gått utan dig. All kärlek!

Noa, min älskade skrutt, du ger mig sådan glädje och kärlek. Jag är så stolt över dig och att få vara din mamma. Nu ska sluta jobba så mycket, nu är det din tid. Så mycket roligt vi ska hitta på, åh vad jag längtar!

Financially supported by FORMAS, Helge Ax.son Johnsons Stiftelse, Wilhelm och Martina Lundgrens vetenskapsfond, Adlerbertska forskningsstiftelsen and Kungl. Vetenskaps- och Vitterhets- Samhället i Göteborg (KVVS).

6. REFERENCES

Abnet, C.C., Tanguay, R.L., Hahn, M.E., Heideman, W., Peterson, R.E. 1999. Two forms of aryl hydrocarbon receptor type 2 in Rainbow trout (*Oncorhynchus mykiss*). J. Biol. Chem. 274, 15159-15166.

Aleksunes, L.M., Klaassen, C.D. 2012. Coordinated regulation of hepatic phase I and II drug-metabolizing genes and transporters using AhR-, CAR-, PXR-, PPARα-, and Nrf2-null mice. Drug Metab. Dispos. 40, 1366-1379.

Al-Salman, F., Plant, N. 2012. Non-coplanar polychlorinated biphenyls (PCBs) are direct agonists for the human pregnane-X receptor and constitutive androstane receptor, and activate target gene expression in a tissue-specific manner. Toxicol. Appl. Pharmacol. 263, 7-13.

Anderson, M.J., Olsen, H., Matsumura, F., Hinton, D.E. 1996. In vivo modulation of 17 beta-estradiol-induced vitellogenin synthesis and estrogen receptor in rainbow trout (*Oncorhynchus mykiss*) liver cells by beta-naphthoflavone. Toxicol. Appl. Pharmacol. 137, 210-218.

Ankley, G.T., Jensen, K.M., Durhan, E.J., Makynen, E.A., Butterworth, B.C., Kahl, M.D., Villeneuve, D.L., Linnum, A., Gray, E.L., Cardon, M., Wilson, V.S. 2005. Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow (*Pimephales promelas*). Toxicol. Sci. 86, 300-308.

Arukwe, A., Goksøyr, A. 1997. Changes in three hepatic cytochrome P450 subfamilies during reproductive cycle in turbot (*Scophthalmus miximus L.*). J. Exp. Zool. 277, 313-325.

Arukwe, A., Goksøyr, A. 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp. Hepatol. 2:4.

Babín, M., Casado, S., Chana, A., Herradón, B., Segner, H., Tarazona, J.V., Navas, J.M. 2005. Cytochrome P4501A induction caused by the imidazole derivative prochloraz in a rainbow trout cell line. Toxicol. In Vitro. 19, 899-902.

Backhaus, T., Faust, M., Scholze, M., Gramatica, P., Vighi, M., Grimme, L.H. 2004. Joint algal toxicity of phenylurea herbicides is equally predictable by concentration addition and independent action. Environ. Toxicol. Chem. 23, 258-264.

Backhaus, T., Faust, M. 2012. Predictive environmental risk assessment of chemical mixtures: A conceptual framework. Environ. Sci. Tech. 46, 2564-2573.

Bainy, A.C.D., Kubota, A, Goldstone, J.V., Lille-Langøy, R., Karchner, S.I., Celander, M.C., Hahn, M.E., Goksøyr, A., Stegeman, J.J. 2013. Functional characterization of a full length pregnane X receptor, expression in vivo, and identification of PXR alleles, in Zebrafish (*Danio rerio*). Aquat. Toxicol. 142-143, 447-457.

Basu, N., Billiard, S., Fragoso, N., Omoike, A., Tabash, S., Brown, S., Hodson, P. 2001. Ethoxyresorufin-*O*-Deethylase induction in trout exposed to mixtures of polycyclic aromatic hydrocarbons. Environ. Toxicol. Chem. 20, 1244-1251.

Baumann, L., Knörr, S., Keiter, S., Nagel, T., Segner, H., Braunbeck, T. 2014. Prochloraz causes irreversible masculinization of zebrafish (*Danio rerio*). Environ. Sci. Pollut. Res. DOI 10.1007/s11356-014-3486-3.

Beischlag, T.V., Morales, J.L., Hollingshead, B.D., Perdew, G.H. 2008. The aryl hydrocarbon receptor complex and the control of gene expression. Crit. Rev. Eukaryot. Gene. Expr. 18, 207-250.

Belenguer, V., Martinez-Capel, F., Masiá, A., Picó, Y. 2014. Patterns of presence and concentration of pesticides in fish and waters of the júcar river (Eastern Spain). J. Hazard. Mater. 265, 271-279.
Bello, S.M., Franks, D.G., Stegeman, J.J., Hahn, M.E. 2001. Acquired resistance to Ah receptor agonists in a population of Atlantic killifish (*Fundulus heteroclitus*) inhabiting a marine superfund site: *in vivo* and *in vitro* studies on the inducibility of xenobiotic metabolizing enzymes. Toxicol. Sci. 60, 77-91.
Bemanian, V., Male, R., Goksøyr, A. 2004. The aryl hydrocarbon receptor-mediated disruption of vitellogenin synthesis in the fish liver: Cross-talk between AHR- and ERα–signaling pathways. Comp. Hepato. 3:2
Bergman, Å., Heindel, J.J., Jobling, S., Kidd, K.A., Zoeller, T.R. 2013. State of the science of endocrine disrupting chemicals – 2012. United Nations Environment Programme and the World Health Organization
Berry, M.N., Friend, D.S. 1969. High-yield preparation of isolated rat liver parenchymal cells. J. Cell Biol. 43, 506-520.
Beyer, A., Biziuk, M. 2009. Environmental fate and global distribution of polychlorinated biphenyls. Rev. Environ. Contamin. Toxicol. 201, 137-158.
Black, D.E., Gutjahr-Gobell, R., Pruell, R.J., Bergen, B., Mills, L., McElroy, A.E. 1998. Reproduction and polychlorinated biphenyls in *Fundulus heteroclitus* (Linnaeus) from New Bedford Harbor, Massachusetts, USA. Environ. Toxicol. Chem. 17, 1405-1414.
Bondesson, M., Hao, R., Lin, C.Y., Williams, C., Gustafsson, J.Å. 2014. Estrogen receptor signaling during vertebrate development. Biochim. Biophys. Acta. doi: 10.1016/j.bbagrm.2014.06.005.
Boxall, A.B., Rudd, M.A., Brooks, B.W., Caldwell, D.J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J.P., Verslycke, T., Ankley, G.T., Beazley, K.F., Belanger, S.E., Berninger, J.P., Carriquiriborde, P., Coors, A., Deleo, P.C., Dyer, S.D., Ericson, J.F., Gagne, F., Giesy, J.P., Gouin, T., Hallstrom, L., Karlsson, M.V., Larsson, D.G., Lazorchak, J.M., Mastrocco, F., McLaughlin, A., McMaster, M.E., Meyerhoff, R.D., Moore,R., Parrott, J.L., Snape, J.R., Murray-Smith, R., Servos, M.R., Sibley, P.K., Straub, J.O., Szabo, N.D., Topp, E., Tetreault, G.R., Trudeau, V.L. and Van Der Kraak, G. 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? Environ. Health Perspect. 120, 1221-1229.
Bresolin, T., de Freitas Rebelo, M., Bainy, A.C.D. 2005. Expression of PXR, CYP3A and MDR1 genes in liver of zebrafish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 140, 403-407.
Budinsky, R.A., Schrenk, D., Simon, T., Vand den Berg, M., Reichard, J.F., Silkworth, J.B., Aylward, L.L., Brix, A, Gasiewicz, T., Kaminski, N., Perdew, G., Starr, T.B., Walker, N.J., Rowlands, J.C. 2014. Mode of action and dose-response framework analysis for receptor-mediated toxicity: The aryl hydrocarbon receptor as a case study. Crit. Rev. Toxicol. 44, 83-119.
Burkina, V., Zlabek, V., Zamaratskaia, G. 2013. Clotrimazole but not dexamethasone, is a potent in vitro inhibitor of cytochrome P450 isoforms CYP1A and CYP3A in rainbow trout. Chemosphere 92, 1099-1104.
Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G.V., Cohen, S., Di Giulio, R.T., Evans, D.H., Gómez-Chiarri, M., Hahn, M.E., Hoover, C.A., Karchner, S.I., Katoh, F., MacLatchy, D.L., Marshall, W.S., Meyer, J.N., Nacci, D.E., Oleksiak, M.F., Rees, B.B., Singer, T.D., Stegeman, J.J., Towle, D.W., Van Veld, P.A., Vogelbein, W.K., Whitehead, A., Winn, R.N., Crawford, D.L, 2007. Fundulus as the premier teleost model in

environmental biology: Opportunities for new insight using genomics. Comp. Biochem. Physiol. Part D Genomics Proteomics, 2, 257-286

Burreau, S., Zebühr, Y., Broman, D., Ishaq, R. 2006. Biomagnification of PBDEs and PCBs in food webs from the Baltic Sea and the northern Atlantic ocean. Sci. Tot. Environ. 366, 659-672.

Campbell, W.C. 1990. Benzimidazoles: Veterinary uses. Parasitol. Today. 4.

Carney,S.A., Prasch, A.L., Heideman, W., Peterson, R.E. 2006. Understanding dioxin development toxicity using the Zebrafish model. Birth Def. Res. Part A 76, 7-18.

Castillo, L.E. Ruepert, C., Solis, E. 2000. Pesticide residues in the aquatic environment of banana plantation areas in the North Atlantic zone of Costa Rica. Environ. Toxicol. Chem. 19, 1942-1950.

Cedergreen, N. 2014. Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology. PloS ONE 9

Celander, M. C. 2011. Cocktail effects on biomarker responses in fish. Aquat. Toxicol. 105, 72-7.

Celander, M., Buhler, D.R., Förlin, L., Goksøyr, A., Miranda, C. L., Woodin, B.R., Stegeman, J.J. 1996. Immunochemical relationships of cytochrome P4503A-like proteins in teleost fish. Fish Physiol. Biochem. 15, 323-332.

Celander, M., Förlin, L. 1991. Catalytic activity and immunochemical quantification of hepatic cytochrome P-450 in β-naphtoflavone and isosafrol treated rainbow trout (*Oncorhyncus mykiss*). Fish Physiol. Biochem. 9, 189-197.

Celander, M., Hahn, M.E., Stegeman, J.J. 1996. Cytochromes P450 (CYP) in the Poeciliopsis lucida hepatocellular carcinoma cell line (PLHC-1): dose- and time-dependent glucocorticoid potentiation of CYP1A induction without induction of CYP3A. Arch. Biochem. Biophys. 329, 113-22.

Chambers, J.E., Greim, H., Kendall, R.J., Segner, H., Sharpe, R.M., Van Der Kraak, G. 2014. Human and ecological risk assessment of a crop protection chemical: a case study with the azole fungicide epoxiconazole. Crit. Rev. Toxicol. 44, 176-210.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L. 2008. Interference of endocrine disrupting chemicals with aromataste CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen. Comp. Endocrinol. 155, 31-62

Christen, V., Oggier, D.M., Fent, K. 2009. A microtiter-plate based cytochrome P4503A activity assay in fish cell lines. Environ. Toxicol. Chem. 28, 2632-2638.

Christiansen, S., Scholze, M., Dalgaard, M., Vinggaard, A.M., Axelstad, M., Kortenkamp, A., Hass, U. 2009. Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. Environ. Health Persp. 117, 1839-1846.

Clark, B.W., Matson, C.W., Jung, D., Di Giulio, R.T., 2010. AHR2 mediates cardiac teratogenesis of polycyclic aromatic hydrocarbons and PCB-126 in Atlantic killifish (*Fundulus heteroclitus*). Aquat. Toxicol. 99, 232–240.

Cody, R.P., Bortone, S.A. 1997.Masculinization of Mosquitofish as an indicator of exposure to kraft mill effluent. Bull. Environ. Contam. Toxicol. 58, 429–436

Cooper, P.S., Vogelbein, W.K., Van Veld, P.A. 1999. Altered expression of the xenobiotic transporter P-glycoprotein in liver and liver tumours of mummichog (Fundulus heteroclitus) from a creosote-contaminated environment. Biomarkers 4, 48-58.

Corcoran, J., Winter, M.J., Tyler, C.R. 2010. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. Crit. Rev. Toxicol. 40, 287-304.

Costa, J., Reis-Henriques, M.A., Castro, L.F.C., Ferreira., M. 2012. Gene expression analysis of ABC efflux transporters, CYP1A and GSTα in Nile tilapia after exposure to benzo(a)pyrene. Comp. Biochem. Physiol. Part C 155, 469-482.

Dahlström, M., Mårtensson, L.G.E., Jonsson, P.R., Arnebrant, T., Elwing, H. 2000. Surface active adrenoreceptor compounds prevent the settlement of cyprid larvae of Balanus improvisus. Biofoul. 16, 191-203.

de Hoop, L., Schipper, A.M., Leuven, R.S.E.W., Huijbregts, M.A.J., Olsen, G.H., Smit, M.G.D., Hendriks, A.J. 2011. Sensitivity of polar and temperate marine organisms to oil components. Environ. Sci. Tech. 45, 9017-9023.

Denison, M.S., Nagy, S.R. 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu. Rev. Pharmacol. Toxicol. 43, 309-34.

Denison, M.S., Soshilov, A.A., He, G., DeGroot, D.E., Zhao, B. 2011. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. Toxicol. Sci. 124, 1-22.

Dumontet, C., Jordan, M.A. 2010. Microtubule-binding agents: a dynamic field of cancer therapeutics. Nat. Rev. Drug Discov. 9, 790-803

Dvořák, Z., Vrzal, R., Ulrichová, J., Pascussi, J.M., Maurel, P., Modrianský, M. 2006. Involvement of cytoskeleton in AhR-dependent CYP1A1 expression. Curr. Drug Metab. 7, 301-13.

Elskus, A.A., Stegeman, J.J., Susani, L.C., Black, D., Pruell, R.J., Fluck, S.J. 1989. Polychorinated biphenyls concentration and cytochrome P450E expression in winter flounder from contaminated environments. Marine Environ. Res. 28, 25-30.

Erickson, M.D., Kaley, R.G. 2011. Applications of polychlorinated biphenyls. Environ. Sci. Pollut. Res. 18, 135-151.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L.H. 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquat. Toxicol. 63, 43-63.

Fent, K., Weston, A.A., Caminada, D. 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122-159.

Ferreira, M., Santos, P., Rey-Salgueiro, L., Zaja, R., Reis-Henriques, A., Smital, T. 2014. The first demonstration of CYP1A and the ABC protein(s) gene expression and activity in European seabass (*Dicentrarchus labrax*) primary hepatocytes. Chemosphere 100, 152-159.

Fick J., Söderström, H., Lindbert, R.H., Phan, C., Tysklind, M., Larsson, J.D.G. 2009. Contamination of surface, ground, and drinking water from pharmaceutical production. Environ. Toxicol. Chem. 28, 2522-2527.

Fick, J., Lindberg, R. H., Kaj, L., Brorström-Lundén, E. 2011. Results from the Swedish national screening programme 2010 Subreport 3. Pharmaceuticals. IVL B2014.

Fleming, C.R., Billiard, S.M., Di Giulio, R.T. 2009. Hypoxia inhibits induction of aryl hydrocarbon receptor activity in topminnow hepatocarcinoma cells in an ARNT-dependent manner. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 150, 383-389.

Galigniana, M.D., Scruggs, J.L., Herrington, J., Welsh, M.J., Carter-Su, C., Housley, P.R., Pratt, W.B. 1998. Heat shock protein 90-dependent (Geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. Mol. Endocrin. 12, 1903-1913.

Galus, M., Jeyaranjaan, J., Smith, E., Li, H., Metcalfe, C., Wilson, J.Y. 2013. Chronic effects of exposure to a pharmaceutical mixture and municipal wastewater in zebrafish. Aquat. Toxicol. 132-133, 212-222.

Gao, H., Dahlman-Wright, K. 2011. The gene regulatory networks controlled by estrogens. Mol. Cell. Endocrinol. 334, 83-90.

Gerbal-Chaloin, S., Pichard-Garcia, L., Fabre, J.M., Sa-Cunha, A., Poellinger, L., Maurel, P., Daujat-Chavanieu, M. 2006. Role of CYP3A4 in the regulation of the aryl hydrocarbon receptor by omeprazole sulphide. Cell. Sign. 18, 740-750

Goksøyr, A., Förlin, L. 1992. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. Aquat. Toxicol. 22, 287-312.

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jönsson, M.E., Nelson, D.R., Stegeman, J.J. 2010. Identification and developmental expression of the full complement of cytochrome P450 genes in Zebrafish. BMC Genomics 11, 643.

Gouin, T., Mackay, D., Jones, K.C., Harner, T., Meijer, S.N. 2004. Evidence for the ”grasshopper” effect and fractionation during long-range atmospheric transport of organic contaminants. Environ. Pollut. 128, 139-148.

Guengerich, P.F. 1999. Cytochrome P-450 3A4: Regulation and role in drug metabolism. Annu. Rev. Pharmacol. Toxicol. 39, 1-17.

Guengerich, P.F. 2001. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem. Res. Toxicol. 14

Gunawickrama, S.H.NP., Aarsæther, N., Orbea, A., Cajaraville, M.P., Goksøyr, A. 2008. PCB77 (3,3´4,4´-tetrachlorobiphenyl) co-exposure prolongs CYP1A induction, and sustains oxidative stress in B(a)P-exposed turbot, *Scophthalmus maximus*, in a long-term study. Aquat. Toxicol. 89, 65-74

Gährs, M., Roos, R., Andersson, P.L., Schrenk, D. 2013. Role of the nuclear xenobiotic receptors CAR and PXR in induction of cytochromes P450 by non-dioxinlike PCBs in rat hepatocytes. Toxicol. Appl. Pharmacol. 272, 77-85.

Hadrup, N., Taxvig, C., Pedersen, M., Nellemann, C., Hass, U., Vinggaared, A.M. 2013. Concentration addition, independent action and generalized concentration addition models for mixture effect prediction of sex hormone synthesis *in vitro*. PLoS ONE. 8

Hahn, M.E., Lamb, T.M., Schultz, M.E., Smolowitz, R.M., Stegeman, J.J. 1993. Cytochrome P4501A induction and inhibition by 3,3´,4,4´-tetrachlorobiphenyl in an Ah receptor- containing fish hepatoma cell line (PLHC-1). Aquat. Toxicol. 26, 185-208.

Hahn, M.E. 2002. Biomarkers and bioassays for detecting dioxin-like compounds in the marine environment. Sci. Tot. Environ. 289, 49-69.

Halling- Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lutzhoft, H.C., Jorgensen, S.E. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment--a review. Chemosphere 36, 357-393.

Hansson, M.C., Wittzell, H., Persson, K., von Schantz, T. 2003. Characterization of two distinct aryl hydrocarbon receptor (AhR2) genes in Atlantic salmon (*Salmo salar*) and evidence for multiple AhR2 gene lineages in salmonid fish. Gene 303, 197-206.

Harrell, J.M., Murphy, P.J.M., Morishima, Y., Chen, H., Mansfield, J.F., Galigniana, M.D., Pratt, W.B. 2004. Evidence for glucocorticoid receptor transport on microtubules by dynein. J. Biol. Chem. 279, 54647-54654.

Harris, C.A., Hamilton, PB., Runnalls, T.J., Vinciotti, V., Henshaw, A., Hodgson, D., Coe, T.S., Jobling, S., Tyler, C.R., Sumpter, J.P. 2011. The consequences of feminization in breeding groups of wild fish. Environ. Health Perspec. 119, 306-311.

Hass, U., Scholze, M., Christiansen, S., Dalgaard, M., Vinggaard, A.M., Axelstad, M., Broeng Metzdorff, S., Kortenkamp, A. 2007. Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. Environ. Health Persp. 115, 122-128.

Hasselberg, L., Meier, S., Svardal, A., Hegelund, T., Celander, M.C. 2004. Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (*Gadus morhua*). Aquat. Toxicol. 67, 303-313.

Hasselberg, L., Grosvik, B.E., Goksøyr, A., Celander, M.C. 2005. Interactions between xenoestrogens and ketoconazole on hepatic CYP1A and CYP3A, in juvenile Atlantic cod (*Gadus morhua*). Comp. Hepatol. 4: 2.

Hasselberg, L., Westerberg, S., Wassmur, B., Celander, M.C. 2008. Ketoconazole, an antifungal imidazole, increases the sensitivity of rainbow trout to 17alpha-ethynylestradiol exposure. Aquat. Toxicol. 86, 256-264.

Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5-17.

Hegelund, T. 2003. Characterization of CYP3A in fish–expression, inhibition, induction and analyses of gene evolution. PhD Thesis. University of Gothenburg. ISBN 91-628-5695-2.

Hegelund, T., Celander, M.C. 2003. Hepatic versus extrahepatic expression of CYP3A30 and CYP3A56 in adult killifish (*Fundulus heteroclitus*). Aquat. Toxicol. 64, 277-291.

Hegelund, T., Ottosson, K., Radinger, M., Tomberg, P., Celander, M.C. (2004). Effects of the antifungal imidazole ketoconazole on CYP1A and CYP3A in rainbow trout and killifish. Environ. Toxicol. Chem. 23, 1326-1334.

Hemmer, M.J., Courtney, L.A., Ortego, L.S. 1995. Immunohistochemical detection of P-glycoprotein in teleost tissue using mammalian polyclonal and monoclonal antibodies. J. Experiment. Zool. 272, 66-77.

Henry, M.J., Sisler, H.D. 1984. Effects of sterol biosynthesis-inhibiting (SBI) fungicides on cytochrome P-450 oxygenations in fungi. Pestic. Biochem. Physiol. 22, 262-275.

Hermens, J., Leeuwangh, P., Musch, A. 1985. Joint toxicity of mixtures of groups of organic aquatic pollutants to the guppy (*Poecilia reticulata*). Ecotoxicol. Environ. Safety. 9, 321-326.

Hestermann, E.V., Stegeman, J.J., Hahn, M.E. 2000. Relative contributions of affinity and intrinsic efficacy to aryl hydrocarbon receptor ligand potency. Toxicol. Appl. Pharmacol. 168, 160-172.

Holt, M.S. 2000. Source of chemical contaminants and routes into the freshwater environment. Food Chem. Toxicol. 38, 21-27.

Houde, M., Murir, D.C.G., Kidd, K.A., Guildford, S., Drouillard, K., Evans, M.S., Wang, X., Whittle, M.D., Haffner, D., Kling, H. 2008. Influence of lake characteristics on the biomagnification of persistent organic pollutants in lake trout food webs. Environ. Toxicol. Chem. 27, 2169-2178.

Huang, S.M., Hall, S.D., Watkins, P., Love, L.A., Serabjit-Singh, C., Betz, J.M., Hoffman, F.A., Honig, P., Coates, P.M., Bull, J., Chen, S.T., Kearns, G.L., Murray, M.D. 2004. Drug interactions with herbal products and grapefruit juice: a conference report. Clin. Pharmacol. Ther. 75, 1-12.

Hutchinson, T.H., Ankley, G.T., Segner, H., Tyler, C.R. 2006. Screening and testing for endocrine disruption in fish—biomarkers as "signposts", not "traffic lights," in risk assessment. Environ. Health Perspect. 114, 104-114

Hylland, K. 2006. Polycyclic aromatic hydrocarbons (PAH) ecotoxicology in marine ecosystem. J. Toxicol. Environ. Health. Part A. 69, 109-123.

Jacobs, M.N., Nolan, G.T., Hood, S.R. 2005. Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). Toxicol. Appl. Pharmacol. 209, 123-133.

Jin, H., Audus, K.L. 2005. Effect of bisphenol A on drug efflux in BeWo, a human trophoblast-like cell line. Placenta. 26, Suppl. A, 96-103.

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R., Sumpter, J.P. 2002. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. Biol. Reprod. 67, 515-524.

Jönsson, M.E., Franks, D.G., Woodin, B.R., Jenny, M.J., Garrick, R.A., Behrendt, L., Hahn, M.E., Stegeman, J.J. 2009. The tryptophan photoproduct 6-formylindolo[3,2-b]carbazole (FICZ) binds multiple AHRs and induces multiple CYP1 genes via AHR2 in zebrafish. Chemico Biolog. Interac. 181, 447-454

Karchner, S.I., Powell, W.H., Hahn, M.E. 1999. Identification and functional characterization of two highly divergent aryl hydrocarbon receptors (AHR1 and AHR2) in the teleost *Fundulus heteroclitus*. J. Biol. Chem. 474, 33814-33824

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U S A. 104, 8897-8901.

Kienzler, A., Berggren, E., Bessems, J., Bopp, S., van der Linden, S., Worth, A. 2014. JRC Science and Policy Reports. Report to the European Commission. Assessment of mixtures – a review of regulatory requirements and guidance. doi:10.2788/84264.

Kim, R.B. 2002. Drugs as P-glycoprotein substrates, inhibitors, and inducers. Drug. Metab. Rev. 34, 47-54.

King-Heiden, T.C., Mehta, V., Xiong, K.M., Lanham, K.A., Antkiewicz, D.S., Ganser, A., Heideman, W., Peterson, R.E. Reproductive and development toxicity of dioxin in fish. Mol. Cell. Endocrinol. 354, 121-138

Kleinow, K.M., Melancon, M.J., Lech, J.J. 1987. Biotransformation and induction: Implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. Environ. Health Perspec. 71, 105-119.

Kleinow, K.M., Nichols, J.W., Hayton, W.L., McKim, J.M., Barron, M.G. 2008. Toxicokinetics in fishes. The Toxicology of Fishes. R. D. G. a. D. Hinton. Washington DC, Taylor and Francis Publishers, 55-152.

Kliewer, S.A. 2003. The Nuclear Pregnane X Receptor Regulates Xenobiotic Detoxification. J. Nutr. 133, 2444-2447.

Kliewer, S.A., Moore, J.T., Wade, L., Staudinger, J.L., Watson, M.A., Jones, S.A., McKee, D.D., Oliver, B.B., Willson, T.M., Zetterstrom, R.H., Perlmann, T., Lehmann, J.M. 1998. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell 92, 73-82.

Klinge, C.M., Bowers, J.L., Kulakosky, P.C., Kamboj, K.K., Swanson, HI. 1999. The aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT) heterodimer interacts with naturally occurring estrogen response elements. Mol. Cell Endocrinol. 157, 105-119.

Klingenberg, M. 1958. Pigments of rat liver microsomes. Arch. Biochem. Biophys. 75, 376-386.
Koop, H., Arnold, R. 1991. Long-term maintenance treatment of reflux esophagitis with omeprazole. Prospective study in patients with H_2-blocker-resistant esophagitis. Diges. Diseas. Sci. 36, 552-557.
Korashy, H.M., Shayeganpour, A., Brocks, D.R., El-Kadi, A.O.S. 2007. Induction of cytochrome P450 1A1 by ketoconazole and intraconazole but not fluconazole in murine and human hepatoma cell lines. Toxicol. Sci. 97, 32-43.
Kortenkamp, A., Faust, M. 2010. Combined exposures to anti-androgenic chemicals: steps towards cumulative risk assessment. Internat. J. Andrology. 33, 463-474.
Kortenkamp, A. 2014. Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment. Curr. Opin. Pharmacol. 19, 105-111.
Krasowski, M.D., Ai, N., Hagey, L.R., Kollitz, E.M., Kullman, S.W., Reschly, E.J., Ekins, S. 2011. The evolution of farnesoid X, vitamin D, and pregnane X receptors: insights from the greenspotted pufferfish (*Tetraodon nigriviridis*) and other non-mammalian species. BMC Biochem. 12
Kreuger, J. 1998. Pesticides in stream water within an agricultural catchment in southern Sweden, 1990-1996. Sci. Tot. Environ. 216, 227-251.
Kubota, A., Goldstone, J.V., Lemaire, B., Takata, M., Woodin, B.R., Stegeman, J.J. 2014. Pregnane X receptor and aryl hydrocarbon receptor both are involved in transcriptional regulation of pxr, CYP2 and CYP3 genes in developing zebrafish. Toxicol. Sci. In press. pii: kfu240. [Epub ahead of print].
Kumagi, T., Suzuki, H., Sasaki, T., Sakaguchi, S., Miyairi, S., Yamazoe, Y., Nagata, K. 2012. Polycyclic aromatic hydrocarbons activate CYP3A4 gene transcription through human pregnane X receptor. Drug. Metab. Pharmacokinet. 25, 200-206.
Kümmerer, K. 2009. The presence of pharmaceuticals in the environment due to human use--present knowledge and future challenges. J. Environ. Manage. 90, 2354-2366.
Kurelec, B. 1992. The multixenobiotic resistance mechanism in aquatic organisms. Crit. Rev. Toxicol. 22, 23-43.
Laetz, C.A., Baldwin, D.H., Collier, T.K., Hebert, V., Stark, J.D., Scholz, N.L. 2009. The synergistic toxicity of pesticides mixtures: Implications for risk assessment and the conservation of endangered Pacific salmon. Environ. Health Persp. 117, 348-353.
Lange, A., Katsu, Y., Miyagawa, S., Ogino, Y., Urushitani, H., Kobayashi, T., Hirai, T., Shears, J.A., Nagae, M., Yamamoto, J., Ohnishi, Y., Oka, T., Tatarazako, N., Ohta, Y., Tyler, C.R., Iguchi, T. 2011. Comparative responsiveness to natural and synthetic estrogens of fish species commonly used in the laboratory and field monitoring. Aquat. Toxicol. 109, 250-258.
Lake, J.L., McKinney, R., Lake, C.A., Osterman, F.A., Heltshe, J. 1995. Comparisons of patterns of polychlorinated biphenyl congeners in water, sediment, and indigenous organisms from New Bedford harbor, Massachusetts. Arch. Environ. Contam. Toxicol. 29, 207-220.
Larsson, D.G.J., Förlin, L. 2002. Male-biased sex ratios of fish embryos near a pulp mill: temporary recovery after a short-term shutdown. Environ. Health. Perspec. 110, 739-742.
Larson, D.G.J., Adolfsson-Erici, M., Parkkonen, J., Petterson, M., Berg, A.H., Olsson, P.E., Förlin, L. 1999. Ethinyloestradiol - an undesired fish contraceptive? Aquat. Toxicol. 45, 91-97.

Larsson, D.G.J., de Pedro, C., Paxeus, N. 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. J. Hazard Mater. 148, 751-755.

Laub, L.B., Jones, M.D., Powell, W.H. 2010. Responsiveness of a Xenopus laevis cell line to the aryl hydrocarbon receptor ligands 6-formylindolo[3,2 b]carbazole (FICZ) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Chemico Biolog. Interac. 183, 202-211.

Lennquist, A., Celander, M.C., Förlin, L. 2008. Effects of medetomidine on hepatic EROD activity in three species of fish. Ecotoxicol. Environ. Safety. 69, 74-79.

Leslie, E.M., Deeley, R.G., Cole, S.P. 2005. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol. Appl. Pharmacol. 204, 216-237.

Levine, S.L., Czosnyka, H., and Oris, J.T. 1997. Effect of the fungicide clotrimazole on the bioconcentration of benzo[a]pyrene in gizzard shad (*Dorosoma cepedianum*): In vivo and in vitro inhibition of cytochrome P4501A activity. Environ. Toxicol. Chem. 16, 306-311.

Levine, S.L., Oris, J.T. 1999. Noncompetitive mixed-type inhibition of rainbow trout CYP1A catalytic activity by clotrimazole. Comp. Biochem. Physiol. Part C. 122, 205-210

Lindberg, R.H., Fick, J., Tysklind, M. 2010. Screening of antimycotics in Swedish sewage treatment plants – Water and sludge. Water Research. 44, 649-657.

Livingstone, D.R. 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 120, 43-49.

Long, M., Laier, P., Vinggaard, A.M, Andersen, H.R., Lynggaard, J., Bonefeld-Jørgensen, E.C. 2003. Effects of currently used pesticides in the AhR-CALUX assay: comparison between the human TV101L and the rat H4IIE cell line. Toxicol. 194, 77-93.

Luckenbach, T., Fischer, S., Sturm, A. 2014. Current advances on ABC drug transporters in fish. Comp. Biochem. Physiol. Part C., 165, 28-52.

Maglich, J.M., Stoltz, C.M., Goodwin, B., Hawkins-Brown. D., Moore, J.T., Kliewer, S.A. 2002. Nuclear pregnane X receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. Mol. Pharmacol. 62, 638-646.

Maher, J.M., Cheng, X., Slitt, A.L., Dieter, M.Z., Klaassen., C.D. 2005. Induction of the multidrug resistance-associated protein family of transporters by chemical activators of receptor-mediated pahtways in mouse liver. Drug. Metab. Dispos. 33, 956-962.

Matthews, J., Gustafsson, J.A. 2006. Estrogen receptor and aryl hydrocarbon receptor signaling pathways. Nucl. Recept. Signal. 4

McArthur, A.G., Hegelund, T., Cox, R.L., Stegeman, J.J., Liljenberg, M., Olsson, U., Sundberg, P., Celander, M.C. 2003. Phylogenetic analysis of the cytochrome P450 3 (CYP3) gene family. J. Mol. Evol. 57, 200-211.

McMillan, B.J., Bradfield, C.A. 2007. The aryl hydrocarbon receptor sans xenobiotics: Endogenous function in genetic model system. Mol. Pharmacol. 72, 487-498.

Medical Products Agency Sweden, 2013. Tillsynsrapport från läkemedelsverket. Godkända läkemedel. Dnr 6.2.11-2014- 029254.

Miller, V.P., Stresser, D.M., Blanchard, A.P., Turner, S., Crespi, C.L. 2000. Fluorometric high-throughput screening for inhibitors of cytochrome P450. Ann. N.Y. Acad. Sci. 919, 26-32.

Mortensen, A.S., Arukwe, A. 2007. Interactions between estrogen- and Ah-receptor signaling pathways in primary culture of salmon hepatocytes exposed to nonylphenol and 3,3',4,4'-tetrachlorobiphenyl (congener 77). Comp. Hepatol. 6:2.
Mortensen, A.S., Tolfsen, C.C., Arukwe, A. 2006. Gene expression patterns in estrogen (nonylphenol) and aryl hydrocarbon receptor agonists (PCB-77) interaction using rainbow trout (*Oncorhynchus Mykiss*) primary hepatocyte culture. J. Toxicol. Environ. Health A 69, 1-19.
Moschet, C., Wittmer, I., Simovic, J. Junghans, M., Piazzoli, A., Singer, H., Stamm, C., Leu, C., Hollender, J. 2014. How a complete pesticide screening changes the assessment of surface water quality. Environ. Sci. Technol. 48, 5423-5432.
Nacci, D., Coiro, L., Champlin, D., Jayaraman, S., McKinney, R., Gleason, T.R., Munns, W.R., Specker, J.L., Cooper, K.R. 1999. Adaptations of wild populations of the estuarine fish *Fundulus heteroclitus* to persistent environmental contaminants. Mar. Biol. 134, 9-17.
Nacci, D.E., Champlin, D., Jayaraman, S. 2010. Adaptation of the estuarine fish *Fundulus heteroclitus* (Atlantic killifish) to polychlorinated biphenyls (PCBs). Estuaries and Coasts 33, 853–864.
Nagahama, Y. 1994. Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38, 217-229.
Nakata, K., Tanaka, Y., Nakano, T., Adachi, T., Tanaka, H., Kaminuma, T., Ishikawa, T. 2006. Nuclear receptor-mediated transcriptional regulation in Phase I, II, and III xenobiotic metabolizing systems. Drug Metab. Pharmacokinet. 21, 437-457.
Navas, J.M., Segner, H. 2000. Antiestrogenicity of β-naphthoflavone and PAHs in cultured rainbow trout hepatocytes: evidence for a role of the aryl hydrocarbon receptor. Aquat. Toxicol. 51, 79-92.
Navas, J.M., Chana, A., Herradon, B., Segner, H. 2004. Induction of cytochrome P4501A (CYP1A) by clotrimazole, a non-planar aromatic compound. Computational studies on structural features of clotrimazole and related imidazole derivatives. Life Sci. 76, 699-714.
Nebert, D.W. 1994. Drug-metabolizing enzymes in ligand-modulated transcription. Biochem. Pharmacol. 47, 25-37.
Nebert, D.W., Russel, D.W. 2002. Clinical importance of the cytochromes P450. Lancet. 360, 1155-1162
Neff, J.M., Stout, S.A., Gunster, D.G. 2005. Ecological risk assessment of polycyclic aromatic hydrocarbons in sediments: identifying sources and ecological hazard. Integr. Environ. Assess. Manag. 1, 22-33
Nelson, D.R. 2006. Cytochrome P450 nomenclature, 2004. Methods Mol. Biol. 320, 1-10.
Nelson, W.G., Bergen, B.J. 2012. The New Bedford Harbor Superfund site long-term monitoring program (1993–2009). Environ. Monit. Assess. 184, 7531–7550
Nichols, J.W., Bonnell, M., Dimitrov, S.D., Escher, B.I., Han, X., Kramer, N.I. 2009. Bioaccumulation assessment using predictive approaches. Integr. Environ. Assess. Manag. 5, 577-597.
Oleksiak, M.F., Karchner, S.I., Jenny, M.J., Franks, D.G., Welch, D.B., Hahn, M.E. 2011. Transcriptomic assessment of resistance to effects of an aryl hydrocarbon receptor (AHR) agonist in embryos of Atlantic killifish (*Fundulus heteroclitus*) from a marine Superfund site. BMC Genomics 12, 263.
Omura, T., Sato, R. 1962. A new cytochrome in liver microsomes. J. Biol. Chem. 237, 1375-1376.

Orton, F., Ermler, S., Kugathas, S., Rosivatz, E., Scholze M., Kortenkamp, A. 2014. Mixture effects at very low doses with combinations of anti-androgenic pesicides, antioxidants, industrial pollutants and chemicals used in personal care products. Toxicol. Appl. Pharmacol. 278, 201-208.

Ortiz de Montellano, P.R. 2010. Hydrocarbon hydroxylation by cytochrome P450 enzymes. Chem. Rev. 110, 932-948.

Pajor, A.M., Stegeman, J.J., Thomas, P., Woodin, B.R. 1990. Feminization of the hepatic microsomal cytochrome P-450 system in Brook trout by estradiol, testosterone, and pituitary factors. J. Exp.Zool. 253, 51-60

Pascussi, J.M., Jounaidi, Y., Drocourt, L., Domergue, J., Balabaud, C., Maurel, P., Vilarem, M.J. 1999. Evidence for the presence of a functional pregnane X receptor response element in the CYP3A7 promoter gene. Biochem. Biophys. Research Commun. 260, 377-381.

Pascussi, J.M., Gerbal-Chaloin, S., Drocourt, L., Assenat, E., Larrey, D., Pichard-Garcia, L., Vilarem, M.J., Maurel, P. 2004. Cross-talk between xenobiotic detoxification and other signaling pathways: clinical and toxicological consequences. Xenobiotica 34, 633-664.

Parks, L.G., Lambright, C.S., Orlando, E.F., Guillette, L.J., Ankley, G.T., Gray, L.E. 2001. Masculinization of female mosquitofish in kraft mill effluent- contaminated fenholloway river water is associated with androgen receptor agonist activity. Toxicol. Sci. 62, 257-267.

Peakall, D.B., Walker, C.H. The role of biomarkers in environmental assessment (3). Vertebrates. Ecotoxicol. 3, 173-179.

Pesonen, M., Andersson, T. 1991. Characterization and induction of xenobiotic metabolizing enzyme activities in a primary culture of rainbow trout hepatocytes. Xenobiot. 21, 461-471.

Petrulis, J.R., Perdew, G.H. 2002. The role of chaperone proteins in the aryl hydrocarbon receptor core complex. Chemico. Biologic. Interac. 141, 25-40

Pollenz, R.S., Sullivan, H.R., Holmes, J., Necela, B., Peterson, R.E. 1996. Isolation and expression of cDNAs from rainbow trout (Oncorhynchus mykiss) that encode two novel basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) proteins with distinct functions in the presence of the aryl hydrocarbon receptor. J. Biol. Chem. 271, 30886-30896.

Powell, W.H., Karchner, S.I., Bright, R., Hahn. M.E. 1999. Functional diversity of vertebrate ARNT proteins: Identification of ARNT2 as the predominant form of ARNT in the Marine Teleost, *Fundulus heteroclitus.* Arch. Biochem. Biophys. 361, 156-163.

Prasch, A.L., Andreasen, E.A., Peterson, R.E., Heideman, W. 2004. Interactions between 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and hypoxia signaling pathways in zebrafish: Hypoxia decreases responses to TCDD in zebrafish embryos. Toxicol. Sci. 78, 68-77.

Price, R.J., Surry, D., Renwick, A.B., Meneses-Lorente, G., Lake, B.G., Evans, D.C. 2000. CYP isoform induction screening in 96-well plates: use of 7-benzyloxy-4-trifluoromethylcoumarin as a substrate for studies with rat hepatocytes. Xenobiot. 30, 781-795.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P. 1994. Estrogenic effects of effluents from sewage-treatment works. Chem. Ecol. 8, 275-285.

Rannug, A., Rannug, U., Rosenkranz, H.S., Winqvist, L., Westerholm, R., Agurell, E., Grafström, A-K. 1987. Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah Receptor and are likely to be endogenous signal substances. J. Biol. Chem. 262, 15422-15427.
Ring, B., Wrighton, S.A., Mohutsky, M. Reverisble mechanisms of enzyme inhibition and resulting clinical significance. The enzyme kinetics in drug metabolism. Fundamentals and applications. Nagar, S., Argikar, U.A., Tweedie, D.J. Humana Press, Springer. ISBN 978-1-62703-757-0
Rodriguez-Antona, C., Donato, M.T., Boobis, A., Edwards, R.J., Watts, P.S., Castell, J.V., Gomez-Lechon, M.J. 2002. Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. Xenobiot. 32, 505-520.
Ritter, L., Solomon, K., Sibley, P., Hall, K., Keen, P., Mattu, G., Linton, B. 2002. Sources, pathways, and relative risk of contaminants in surface water and groundwater: A perspective prepared for the Walkerton inquiry. J. Toxicol. Environ. Health Part A. 65, 1-142.
Ruus, A., Aarre Daae, I., Hylland, K. 2012. Accumulation of polychlorinated biphenyls from contaminated sediment by Atlantic cod (Gadus morhua): Direct accumulation from resuspended sediment and dietary accumulation via the polychaete nereis virens. Environ. Toxicol. Chem. 31, 2472–2481.
Safe, S. 1992. Toxicology, structure-function relationship, and human and environmental health impacts of polychlorinated biphenyls: progress and problems. Environ. Health. Perspect. 100, 259-268.
Safe, S., Wormke, M., Samudio, I. 2000. Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells. J. Mammary Gland Biol. Neoplasia 5, 295-306
Sarkar, A., Ray, D., Shrivastava, A.N, Sarker, S. 2006. Molecular Biomarkers: their significance and application in marine pollution monitoring. Ecotoxicol, 15, 333-340.
Schlenk, D., George, S., James, M., Hurk, P.V.D., Willet, K., Kullman, S., Celander, M., Gallagher, E. 2008. Biotransformation. The Toxicology of Fishes. R. D. G. a. D. Hinton. Washington DC, Taylor and Francis Publishers: 153-234.
Schlezinger, J.J., White, R., Stegeman, J.J. 1999. Oxidative inactivation of cytochrome P-450 1A (CYP1A) stimulated by 3,3´,4,4´-Tetrachlorobiphenyl: Production of reactive oxygen by vertebrate CYP1As. Mol. Pharmacol. 56, 588-597.
Schmidt, J.V., Bradfield, C.A. 1996. Ah Receptor signaling pathways. Annu. Rev. Cell Dev. Biol. 12, 55-89
Schulz, M., Iwersen-Bergmann, S., Andresen, H., Schmoldt, A. 2012. Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. Crit. Care. 16
Schöller, A., Hong, N.J., Bischer, P., Reiners, J.J. 1994. Short and long term effects of cytoskeleton-disrupting drugs on cytochrome P450 Cyp1a-1 induction in murine hepatoma 1c1c7 cells: Suppression by the microtubule inhibitor. Mol. Pharmacol. 45, 944-954.
Scripture, C.D., Figg, W.D. 2006. Drug interactions in cancer therapy. Nat. Rev. Cancer. 6, 546-558.
Segner, H. 1998. Isolation and primary culture of teleost hepatocytes. Comp. Biochem. Physiol. Part A 120, 71-81.
Shapiro, A.B., Fox, K., Lam, P., Ling, V. 1999. Stimulation of P-glycoprotein-mediated drug transport by prazosin and progesterone. Eur. J. Biochem. 259, 841-850.

Snegaroff, J., Bach, J. 1989. Effects of the fungicide prochloraz on xenobiotic metabolism in rainbow trout: inhibition *in vitro* and time course of induction *in vivo*. Xenobiotica. 19, 255-267.
Smital, T., Kurelec, B. 1998. The chemosensitizers of multixenobiotic resistance mechanism in aquatic invertebrates: a new class of pollutants. Mutat. Res. 399, 43-53.
Stegeman, J.J., Hahn, M.E. 1994. Biochemistry and molecular biology of monooxygenases: Current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. Aquatic toxicology: molecular, biochemical and cellular perspectives D. C. Malins, Ostrander, G.K., Lewis Publishers 87-206.
Sturm, A., Cravedi, J.P., Perdu, E., Baradat, M., Segner, H. 2001. Effects of prochloraz and nonylphenol diethoxylate on hepatic biotransformation enzymes in trout: a comparative in vitro/in vivo-assessment using cultured hepatocytes. Aquat. Toxicol. 53, 229-245
Sumpter, J.P, Jobling, S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Perspect. 103, Suppl 7, 173-178.
Sundberg, H., Tjärnlund, U., Åkerman, G., Blomberg, M., Ishaq, R., Grunder, K., Hammar, T., Broman, D., Balk, L. 2005. The distribution and relative toxic potential of organic chemicals in a PCB contaminated bay. Marine. Poll. Bull. 50, 195-207.
Swedish Environmental Protection Agency, 2013. Gifter och miljö. ISBN 978-91-620-6569-0
Takeuchi, S., Iida, M., Yabushita, H., Matsuda, T., Kojima, H. 2008. *In vitro* screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and *in vivo* mouse liver cytochrome P450-1A induction by propanil, diuron and linuron. Chemosphere. 74, 155-165.
Tanguay, R.L., Abnet, C.C., Heideman, W., Peterson, R.E. 1999. Cloning and characterization of the zebrafish (*Danio rerio*) aryl hydrocarbon receptor. Biochim. Biophys. Acta. 1444, 35-48.
Tanguay, R.L., Andreasen, E., Heideman, W., Peterson, R.E. 2000. Identification and expression of alternatively spliced aryl hydrocarbon nuclear translocator 2 (ARNT2) cDNAs from zebrafish with distinct functions. Biochim. Biophys. Acta. 1494, 117-128.
Thadani-Mulero, M., Nanus, D.M., Giannakakou, P. 2012. AR on the move; boarding the microtubule expressway to the nucleus. Cancer Res. 15, 4611-4615.
Thibaut, R., Schnell, S., Porte, C. 2009. Assessment of metabolic capabilities of PLHC-1 and RTL-W1 fish liver cell lines. Cell Biol. Toxicol. 25, 611-622.
Thummel, K.E., Wilkinson, G.R. 1998. In vitro and in vivo drug interactions involving human CYP3A. Annu. Rev. Pharmacol. Toxicol. 38, 389-430.
Timme-Laragy, A., Cockman, C.J., Matson, C.W., Di Guilio, R.T. 2007. Synergistic induction of AHR regulated genes in developmental toxicity from co-exposure to two model PAHs in zebrafish. Aquat. Toxicol. 85, 241-250.
Tseng, H.P., Hseu, T.H., Buhler, D.R., Wang, W.D., Hu, C.H. 2005. Constitutive and xenobiotics-induced expression of a novel CYP3A gene from zebrafish larva. Toxicol. Appl. Pharmacol. 205, 247-258.
U.S Environmental Protection Agency 2014. New Bedford Harbor superfund site – Brief summary. SDMS Doc ID 538674.
Vanden Bossche, V., Koymans, L., Moereels, H. 1995. P450 inhibitors of use in medical treatment: focus on mechanisms of action. Pharmac. Ther. 67, 79-100.

Vanden Bossche, H., Engelen, M., Rochette, F. 2003. Antifungal agents of use in animal health-chemical, biochemical and pharmacological aspects. J. Vet. Pharmacol. Therap. 26, 5-29.
Van der Oost, R., Beyer, J., Vermeulen, N.P.E. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57-149.
Van Veld, P.A., Westbrook, D.J. 1995. Evidence for depression of cytochrome P4501A in a population of chemically resistant mummichog (Fundulus heteroclitus). Environ. Sci. 3, 221–234.
Vollrath, V., Wielandt, A.M., Iruretagoyena, M., Chianale, J. 2006. Role of Nrf2 in the regulation of the Mrp2 (ABCC2) gene. Biochem. J. 395, 599-609.
Vrzal, R., Daujat-Chavanieu, M., Pascussi, J.M., Ulrichová, J., Maurel, P., Dvorak, Z. 2008. Microtubules-interfering agents restrict aryl hydrocarbon receptor-mediated CYP1A2 induction in primary cultures of human hepatocytes via c-jun-N-terminal kinase and glucocorticoid receptor. Eur. J. Pharmacol. 581, 244-54.
Walter, H., Consolaro, F., Gramatica, P., Schloze, M., Altenburger, R. 2002. Mixture toxicity of priority pollutants at No Observe Effect Concentrations (NOECs). Ecotoxicol. 11, 299-310.
Wassmur, B., Gräns, J., Kling, P., Celander, M.C. 2010. Interactions of pharmaceuticals and other xenobiotics on hepatic pregnane X receptor and cytochrome P450 3A signaling pathway in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 100, 91-100.
Wassmur, B. 2012. Detoxification mechanisms in fish: Regulation and function of biotransformation and efflux in fish exposed to pharmaceuticals and other pollutants. PhD Thesis. University of Gothenburg. ISBN 978-91-628-8540-3
Weaver, G. 1984. PCB contamination in and around New Bedford, Mass. Environ. Sci. Technol. 18, 22A-27A.
Weber, R., Watson, A., Forter, M., Oliaei, F. 2011. Persistent organic pollutants and landfills- a review of past experiences and future challenges. Waste Managem. Res. 29, 107-121.
White, L.D., Cory-Slechta, D.A., Gilbert, M.E., Tiffany-Castiglioni, E., Zawia, N.H., Virgolini, M., Rossi-George, A., Lasley, S.M., Qian, Y.C., Riyaz Basha, M.D. New and evolving concepts in the neurotoxicology of lead. Contemp. Issues Toxicol. 225, 1-27.
Whitehead, A., Triant, D.A., Champlin, D., Nacci, D. 2010. Comparative transcriptomics implicates mechanisms of evolved pollution tolerance in a killifish population. Molec. Ecol. 19, 5186-5203.
Whyte, J. J., Jung, R. E., Schmitt, C. J. and Tillitt, D. E. 2000. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Crit. Rev. Toxicol. 30, 347-570.
World Health Organization. 2006. Preventing disease through healthy environments. Toward an estimate of the environmental burden of disease. ISBN 92 4 159 382 2.
Yuan, L., Lv, B., Zha, J., Wang, W., Wang, Z. 2014. Basal and benzo[a]pyrene-induced expression profile of phase I and II enzymes and ABC transporter mRNA in the early life stage of Chinese rare minnows (*Gobiocypris rarus*). Ecotoxicol. Environ. Safety 106, 86-94.
Zaja, R., Klobucar, R.S., Smital, T. 2007. Detection and functional characterization of Pgp1 (ABCB1) and MRP3 (ABCC3) efflux transporters in the PLHC-1 fish hepatoma cell line. Aquat. Toxicol. 81, 365-376.